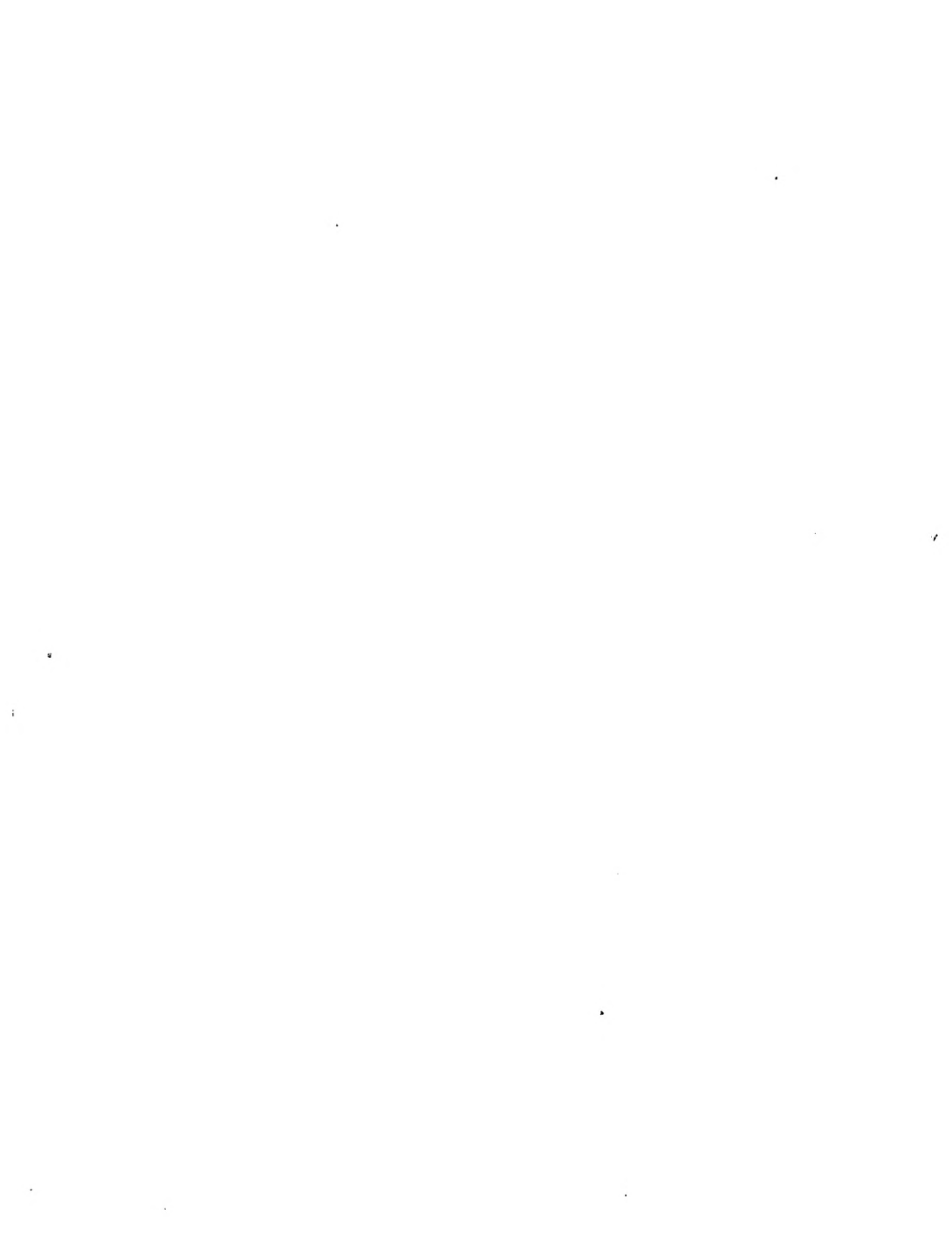# CONTENTS

Effect of hydroxyurea on subcellular activities of thymidine kinase in developing and aging rat brain

V. PRABHAKAR and KALLURI SUBBA RAO*

Neurochemistry Laboratory, School of Life Sciences, University of Hyderabad, Hyderabad 500 134, India

Abstract. Hydroxyurea, when injected intraperitoneally at a dose of 1 mg/g body weight, inhibited thymidine kinase activity in developing rat cerebrum (16-day-embryonic) and cerebellum (7-day-postnatal) within a few hours of administration. The inhibition was time-dependent and both cytosolic and mitochondrial thymidine kinases were affected. Under the same conditions, the activities of certain other enzymes concerned with DNA metabolism, *viz.*, DNA polymerase, and acid and alkaline DNases were not inhibited. Further, the addition of hydroxyurea *in vitro* had no effect on the activity of any of the enzymes studied. However, similar treatment given to 2-year-old rat failed to exert any inhibition on either the mitochondrial or soluble thymidine kinase activities in grey and white matter regions of cerebrum and cerebellum. It is inferred that hydroxyurea, apart from its already known effect on ribonucleotide reductase of replicating cells, also affects thymidine kinase.

Keywords. Hydroxyurea; mitochondrial and cytosolic thymidine kinases; DNA polymerase; acid and alkaline DNases..

Introduction

It is well known that thymidine kinase (ATP: thymidine 5′-phosphotransferase, EC 2·7·1·21) is the principal enzyme converting thymidine to the ultimate precursor (dTTP) for DNA synthesis (Bollum and Potter, 1959). Hence, thymidine kinase activity is widely used as a sensitive marker in following semiconservative DNA synthesis (Bresnick, 1978), although it is an enzyme of the so-called salvage pathway.

Hydroxyurea, a cancer chemotherapeutic drug, is reported to be an inhibitor of replicative DNA synthesis and is known to exert its action by inhibiting ribonucleotide reductase (Young and Hodas, 1964). However, there is abundant evidence in the literature to show that hydroxyurea when added to cell suspensions inhibits thymidine incorporation into DNA (Timson, 1975). No systematic attempt has been made to examine the mechanism by which hydroxyurea is able to achieve such an inhibition. Recent work from this laboratory has revealed, for the first time, that the activity of thymidine kinase is inhibited by hydroxyurea in developing rat cerebellum (Mira Kaplay *et al.*, 1983) and cerebrum (Prabhakar *et al.*, 1984). Further, Yamada *et al.* (1979, 1980) showed that thymidine kinase exists in several forms in rat cerebellum and concluded that the enzyme present in cytosol is associated with nuclear DNA synthesis, while the mitochondrial enzyme, which is the predominant form in

to the age of the brain. It is shown here that hydroxyurea inhibits both soluble and particulate forms of thymidine kinase in different regions of developing but not of old rat brain.

Materials and methods

Materials

[^{3}H-Methyl]-thymidine (15·5 Ci/mmol) was obtained from Bhabha Atomic Research Centre, Bombay. [^{3}H-Methyl] dTTP (46 Ci/mmol) was purchased from the Radio-chemical Centre, Amersham, England.

Hydroxyurea, dithiothreitol, Tris, succinic acid (disodium salt), thymidine, ATP, dATP, dGTP, dCTP, dTTP, 2',3'-cyclic AMP, highly polymerised calf thymus DNA, bovine serum albumin, alkaline phosphatase from *Escherichia coli*, were products of Sigma Chemical Company, St. Louis, Missouri, USA. Whatman DE-81 discs of 2·3 cm diameter and Whatman 42 filter papers (11 cm) were obtained from Whatman Ltd., England. Triton X-100 of scintillation grade was from Koch-Light Laboratories Ltd., Colnbrook Bucks, England. The other chemicals used were of analytical grade.

Animals

Rats of Wistar strain in the age group required were obtained from the University animal house. Animals of both sexes were used.

For the studies with embryonic brain, timed pregnancies were achieved by placing pro-estrus female rats (2–3 month old) in cages overnight (from 4 p.m. to 9·30 a.m.) with males of the same strain. The presence of sperm in the vaginal smears was taken as an indication of successful breeding, and the day was fixed as day zero of gestation. In the experiments with postnatal brain, 6–8 pups were raised with one mother, with the day of birth counted as day one.

Subcellular fractionation and enzyme assay

Brain tissue was homogenized in 5–10 volumes of medium containing 0·25 M sucrose, 0·02 M Tris-HCl buffer, pH 7·4, 4 mM $MgCl_2$ and 1 mM DTT in a motor-driven Potter-Elvehjem homogenizer with a teflon pestle. The mitochondrial and cytosol fractions were prepared according to the procedure of Yamada *et al.* (1979). These two fractions were used as the enzyme source for thymidine kinase assay. The mitochondrial fraction was suspended in a minimal volume of the homogenizing medium containing 1% Triton X-100 (w/v) and was kept frozen overnight in order to facilitate the release of the enzyme (Masui and Garren, 1971).

Acetone powders were prepared according to the method of Morton (1955) from mitochondrial and cytosol fractions of cerebellum, and grey and white matter regions of cerebrum of 2 year old rat brain. The enzyme proteins from each acetone powder

(Prohaska *et al.*, 1973). The inorganic phosphate that was liberated at the end of the reaction was estimated as described by Kyaw *et al.* (1985). The purity of the mitochondrial fraction was checked by measuring succinic dehydrogenase (SDH) activity. SDH activity was determined by a combination of the procedures of Nachlas *et al.* (1960) and Sushecla and Ramasarma (1971) with a few modifications. The reaction mixture contained, in 0·5 ml, 40 mM phosphate buffer, pH 7·6, 17·5 mM succinic acid (disodium salt) and 100 μl of 3 mM 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride and 10 μl of 1% (w/v) phenazine methosulphate. The enzyme initiated reaction was terminated by the addition of 1 ml of glacial acetic acid: tetrachloroethylene (3:1 v/v) after 10 min of incubation at 37°C. The coloured product was eluted into tetrachloroethylene and measured at 500 nm.

DNases were assayed according to the procedure described earlier from this laboratory (Subba Rao and Subba Rao, 1982). The tissues were homogenized in 9 volumes of ice-cold glass-distilled water and the crude homogenates were used for the assay of acid and alkaline DNases.

In order to measure DNA polymerase activity, the tissues were homogenized in 20 volumes of medium containing 0·02 M Tris-HCl buffer, pH 7·5, 0·1 mM β-mercapto-ethanol, 1 mM $MgCl_2$, 0·1 mM EDTA, 5% (w/v) glycerol, 1% (w/v) Triton X-100 and 0·5 M KCl. The homogenates were centrifuged at 100,000 g for 1 h. The supernatant thus obtained was used as the enzyme source. DNA polymerase activity was determined as described by Subba Rao and Subba Rao (1984).

Radioactivity was measured in a Beckman LS-1800 liquid scintillation counter with automatic quench correction facility. Estimation of protein was carried out according to the procedure of Lowry *et al.* (1951).

Results

CNPase activity is generally considered to be a marker activity for myelin and oligodendrocytes (Kurihara and Tsukada, 1967). CNPase activity was determined in white and grey matter of 2-year-old rat cerebrum. The activity was 3 times higher in white matter (5·12±0·19) than in grey matter (1·62±0·13). These values give an indication of the relative purity of the tissues. SDH activity was determined in homogenate and mitochondrial and cytosolic fractions of 7-day cerebellum to check the purity of the fractions. Specific (μ mol INT reduced/h/mg protein) and total activities (mean±SD of 4 experiments) were 0·715±0·037 and 1·267±0·023, 1·91±0·071 and 1·013±0·024, and 0·01±0·009 and 0·022±0·02, respectively. These values show that most of the activity (about 80%) was in the mitochondrial fraction.

The time course of the effect of hydroxyurea on the subcellular activities of thymidine kinase in 16-day-embryonic rat cerebrum is presented in table 1. Thymidine kinase activity in cytosol fraction is inhibited (43%) at 5 h after the injection, and is significantly higher at 9 h. However, at 20 h the activity is once again markedly inhibited (86%). The mitochondrial thymidine kinase, demonstrated to be a separate gene product (Berk and Clayton, 1973) also shows a similar pattern of changes in response to hydroxyurea administration.

Table 2 shows the effect of hydroxyurea treatment on the activities of certain other

of thymidine kinase activity at 8 and 48 h, but not at 24 h the injection (table

Table 1. Time course of effect of hydroxyurea on subcellular activities of thymidin in 16-day-embryonic rat cerebrum.

Time between drug injection and sacrifice (h)	Mitochondria		Cytosol	
	Sp. act.	Total act.	Sp. act.	Total
Control	14.66 ± 2.39	6.07 ± 0.99	110.18 ± 23.09	$69.05 \pm$
5	5.91 ± 1.42^a	1.54 ± 0.52^a	62.96 ± 4.28^a	$40.64 \pm$
9	13.51 ± 2.89	4.08 ± 1.09^b	241.78 ± 35.11^a	$130.31 \pm$
20	2.75 ± 0.49^a	0.78 ± 0.15^a	15.51 ± 3.67^a	$10.67 \pm$

Hydroxyurea was administered intraperitoneally at different times into pregna (1 mg/g body weight), keeping the sacrificing time fixed at 9.30 a.m. on the 16th gestation.

Specific activity is expressed as picomoles of thymidine phosphorylated/min/mg Total activity is expressed as activity per brain region under study. Values are mean A minimum of 6 separate experiments were performed with 2 or more animals p each case.

^{a}Significant difference from control ($P < 0.01$).
^{b}Significant difference from control ($P < 0.05$).

Table 2. Effect of hydroxyurea on the activities of DNases and DNA polymerase i 16-day-embryonic cerebrum.

Time between drug injection and sacrifice (h)	Specific activity		
	Acid DNase	Alkaline DNase	DNA polymerase
Control	9.7 ± 0.91	10.6 ± 2.10	500.0 ± 44.3
5	12.6 ± 0.73^a	11.7 ± 2.0	550.5 ± 40.1
9	9.8 ± 0.65	10.6 ± 2.88	509.7 ± 23.4
20	14.2 ± 0.74^a	13.1 ± 1.19	429.0 ± 59.0

Specific activity of DNases is expressed as μg of acid-soluble DNA phosphorus/2 h/mg protein. Specific activity of DNA polymerase is expressed a picomoles of TMP incorporated/h/mg protein. Values are means $\pm$ SD of a minimum of 4 observations.

^{a}Significant difference from control ($P < 0.02$).

Table 3. Time course of effect of hydroxyurea on subcellular activities of thymidin in 7-day-postnatal rat cerebellum.

Time between drug injection and sacrifice (h)	Mitochondria		Cytosol	
	Sp. act.	Total act.	Sp. act.	Total
Control	14.11 ± 5.17	7.53 ± 2.73	162.50 ± 35.63	$108.25 \pm$
8	9.27 ± 2.16	4.65 ± 1.31	88.99 ± 25.45^a	$44.84 \pm$
24	14.56 ± 2.12	2.78 ± 1.24^a	195.35 ± 37.55	$71.47 \pm$
48	4.67 ± 0.46^a	1.31 ± 0.14^a	88.07 ± 15.58^a	$32.25 \pm$

this case also both mitochondrial and cytosolic thymidine kinase activities were affected in similar fashion. No inhibition of DNA polymerase and acid and alkaline DNases was noticed, nor did *in vitro* addition of hydroxyurea have any effect on thymidine kinase activity (data not shown).

In the next experiment, the relationship between hydroxyurea inhibition of thymidine kinase activity and the age of the brain was examined. The effect of hydroxyurea administration on the mitochondrial and soluble thymidine kinase activities in grey and white matter regions of cerebrum and cerebellum in 2-year-old rat brain was tested. These results are shown in tables 4–6. It may be noted that the

Table 4. Time course of effect of hydroxyurea on subcellular activities of thymidine kinase in grey matter region of 2-year-old rat cerebrum.

Time between drug injection and sacrifice (h)	Mitochondria		Cytosol	
	Sp. act.	Total act.	Sp. act.	Total act.
Control	6·67 ± 1·78	9·14 ± 4·28	0·12 ± 0·11	0·75 ± 0·72
8	5·72 ± 1·09	6·28 ± 2·53	0·15 ± 0·09	0·91 ± 0·59
24	9·63 ± 1·30[a]	13·18 ± 2·21	0·18 ± 0·10	0·56 ± 0·38
48	7·66 ± 2·05	10·21 ± 3·94	0·17 ± 0·04	0·98 ± 0·27

Hydroxyurea was injected intraperitoneally (1 mg/g body weight) at different times prior to a fixed sacrificing time, 10·30 a.m. on the day of sacrifice.
In these experiments with old brain, extracts of acetone powder preparations (materials and methods), were used as enzyme source as fresh enzyme preparations exhibited anomalous kinetics and very low activity, probably because of the presence of high amounts of lipids. Values are mean ± SD of a minimum of 6 independent observations. Other details are as given in table 1.
[a]Significant difference from control ($P < 0.05$).

Table 5. Time course of effect of hydroxyurea on subcellular activities of thymidine kinase in white matter region of 2-year-old rat cerebrum.

Time between drug injection and sacrifice (h)	Mitochondria		Cytosol	
	Sp. act.	Total act.	Sp. act.	Total act.
Control	7·08 ± 1·57	10·75 ± 5·08	0·49 ± 0·16	1·97 ± 0·51
8	6·13 ± 2·17	8·23 ± 4·50	0·70 ± 0·58	3·06 ± 2·22
24	10·03 ± 1·37[a]	14·30 ± 4·58	0·39 ± 0·24	1·42 ± 0·77
48	7·25 ± 1·10	8·74 ± 2·07	0·30 ± 0·27	1·52 ± 1·33

Details are as given in table 4.
[a]Significant difference from control ($P < 0.05$).

soluble thymidine kinase activity in old brain is very low and just detectable. On the other hand, significant thymidine kinase activity is seen in the mitochondrial fraction of old brain. Hydroxyurea exerted no inhibitory effect on thymidine kinase activity

in 2-year-old rat cerebellum.

Time between drug injection and sacrifice (h)	Mitochondria		Cytosol	
	Sp. act.	Total act.	Sp. act.	Total act.
Control	$4 \cdot 08 \pm 1 \cdot 02$	$1 \cdot 19 \pm 0 \cdot 57$	$0 \cdot 46 \pm 0 \cdot 19$	$1 \cdot 05 \pm 0 \cdot 34$
8	$7 \cdot 85 \pm 1 \cdot 17^a$	$2 \cdot 04 \pm 0 \cdot 57$	$0 \cdot 42 \pm 0 \cdot 33$	$0 \cdot 87 \pm 0 \cdot 52$
24	$7 \cdot 26 \pm 0 \cdot 88^a$	$2 \cdot 24 \pm 0 \cdot 22^a$	$0 \cdot 34 \pm 0 \cdot 14$	$0 \cdot 76 \pm 0 \cdot 27$
48	$4 \cdot 84 \pm 3 \cdot 96$	$1 \cdot 48 \pm 1 \cdot 08$	$0 \cdot 22 \pm 0 \cdot 18$	$0 \cdot 56 \pm 0 \cdot 49$

Details are as given in table 4.
[a] Significant difference from control ($P < 0.05$).

Discussion

Three significant observations have arisen out of the present studies: (i) hydroxyurea exerts inhibition *in vivo*, on thymidine kinase activity in regions of developing rat brain; (ii) both mitochondrial and soluble thymidine kinases are affected by hydroxyurea treatment and (iii) in old brain, no inhibitory effect of hydroxyurea on thymidine kinase activity could be seen.

It is observed that the hydroxyurea effect is time-dependent. It is not clear why at certain times after drug treatment (*e.g.*, 9 h in table 1 and 24 h in table 3) the activity of thymidine kinase is either normal or even above normal. It is possible that a proportion of the proliferating cells might have escaped the action of the drug by having already entered S-phase when the drug was administered. Similar anomalous effects of hydroxyurea have been observed by other workers earlier. For example, Rabes *et al.* (1974) found enhanced incorporation of thymidine into DNA in regenerating rat liver after hydroxyurea treatment. It thus appears that time-course studies must be conducted to examine the effects of hydroxyurea on any metabolic event and single-time studies may not give a correct picture.

As hydroxyurea has no effect on thymidine kinase activity *in vitro* (data not shown), it appears that the effect *in vivo* is indirect. It is possible that a metabolite of hydroxyurea rather than hydroxyurea itself, is the cause for such inhibition *in vivo*, particularly because this drug is known to have a very short half-life (Philips *et al.*, 1967). Moreover, hydroxyurea has been shown to be a genotoxic compound after metabolic activation (Andrae and Ziegler–Skylakakis, 1984). It has also been demonstrated that hydroxyurea crosses the blood-brain barrier (Magrath *et al.*, 1974).

The present results also reveal that the effect of hydroxyurea on thymidine kinase may not perhaps be ignored as a non-specific effect since other enzymes concerned with DNA metabolism, *viz.*, DNA polymerase, and acid and alkaline DNases are not inhibited under the same conditions. It is pertinent to mention here that acid DNase (DNase II) has been shown to be an S-phase dependent enzyme in HeLa cells (Slor *et al.*, 1973).

Till now, hydroxyurea has been considered to serve as a tool in the measurement of DNA repair replication by thymidine incorporation after suppressing replicative DNA synthesis (Vilenchik and Tretjak, 1977) which occurs during the S-phase of the cell cycle (Brent, 1971). However, mitochondrial thymidine kinase, which is

Clayton, 1973). It is to be further noted that mitochondrial DNA synthesis occurs not only during the S-phase but also spans the other phases of the cell cycle (Koch and Stockstad, 1967). The present results indicate the sensitivity of both mitochondrial and soluble thymidine kinase activities to hydroxyurea. We have earlier shown this to be the case with regenerating rat liver (Prabhakar *et al.*, 1987). It appears that the target for the action of this drug is at a level common to the production of both mitochondrial and soluble thymidine kinases. The lack of inhibitory effects of hydroxyurea on thymidine kinase in different regions of aging brain may perhaps be taken to indicate that hydroxyurea inhibits thymidine kinase activity only in such cells that are undergoing replication. Thus, the action of this drug is cell-cycle dependent. Further work should clarify this aspect to a greater extent.

Acknowledgements

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Comparative study of conformational behaviour of leucine and methionine enkephalinamides by ^{1}H-nuclear magnetic resonance spectroscopy

M. M. DHINGRA and ANIL SARAN

Chemical Physics Group, Tata Institute of Fundamental Research, Homi Bhabha Road, Bombay 400 005, India

Abstract. The conformational proclivity of leucine and methionine enkephalinamides in deuterated dimethyl sulphoxide has been investigated using proton magnetic resonance at 500 MHz. The resonances from the spin system of the various amino acid residues have been assigned from the 2-dimensional correlated spectroscopy spectra. The temperature variation of the amide proton shifts indicates that none of the amide proton is intramolecularly hydrogen-bonded or solvent-shielded. The analysis of vicinal coupling constants, $^3J_{HN-C^\alpha H}$, along with temperature coefficients and the absence of characteristic nuclear Overhauser effect cross peaks between the NH protons reveal that there is no evidence of the chain folding in these molecules. However, the observation of nuclear Overhauser effect cross peaks between the NH and the $C^\alpha H$ of the preceding residue indicates preference for extended backbone conformation with preferred side chain orientations particularly of Tyr and Phe in both [Leu^5]- and [Met^5]-enkephalinamides.

Keywords. Enkephalinamides; nuclear magnetic resonance; structure-activity relationship; endogenous peptides; temperature coefficients.

Introduction

Leucine and methionine enkephalins (H$_2$N-Tyr^1-Gly^2-Gly^3-Phe^4-Leu^5/Met^5-COOH) are endogenous pentapeptides (Hughes *et al.*, 1975) that have been shown to possess analgesic properties similar to those associated with alkaloid opiates (morphine) and their agonists and antagonists (Belluzi *et al.*, 1976; Bradbury *et al.*, 1976). Since opiates and enkephalins belong to different classes of compounds, there has been a spate of NMR investigations of structure-activity relationships (Roques *et al.*, 1976; Jones *et al.*, 1977; Khaled *et al.*, 1977; Zetta and Cabassi, 1982). The results of such investigations have been reviewed by Schiller (1984). Based on the proton coupling constants and temperature coefficients, a preferred backbone conformation for [Met^5]-enkephalins in hexadeutero dimethyl sulphoxide (DMSO-d$_6$) has been proposed, while on the basis of concentration and temperature dependence of C$^\alpha$-resonances, a conformational equilibrium has been proposed for the same molecule in the same solvent (Garbay-Jaureguiberry *et al.*, 1976; Jones *et al.*, 1976; Higashijima *et al.*, 1979). However, in aqueous solution the conformation of [Met^5]-enkephalin has been a controversial issue. Based on results from one-dimensional nuclear Overhauser (NOE) effect experiments, Gupta *et al.* (1986) have proposed a folded conformation for the molecule but this has been recently refuted by Motta *et al.* (1987). The results of NMR studies on [Leu^5]-enkephalin have suggested the

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Abbreviations used: [Leu^5]-/[Met^5]-enkephalin, Enkephalin with leucine/methionine at position 5;

possibility of a less defined backbone conformation in DMSO-d_6 (Garbay Jaureguiberry et al., 1977; Fischman et al., 1978).

The introduction of D-Ala and D-Ser in place of Gly[2] in [Met[5]]-enkephalin and [Leu[5]]-enkephalin-threonine (a synthetic hexapeptide), respectively, results in relatively more potent analogues with rigid backbones as demonstrated by proton spin-lattice relaxation and 2D NOESY studies (Niccolai et al., 1980; Dhingra and Saran, 1987). Similarly, the amidation of [Met[5]]-enkephalin has also been demonstrated to impart rigidity to the backbone (Gairin et al., 1981) as well as enhanced potency to the peptide (Chang et al., 1976). Comparative conformational studies by NMR on [Met[5]]-enkephalin and [Met[5]]-enkephalinamide in DMSO-d_6 revealed that the native peptide adopts a folded backbone conformation while there is no folding in [Met[5]]-enkephalinamide (Higashijima et al., 1979). Furthermore, a circular dichroism (CD) study on [Met[5]]-enkephalin in methyl alcohol and trifluoroethanol indicated considerable flexibility (Sudha and Balaram, 1981) but no attempt was made to ascertain the nature of conformational changes subsequent to alteration in solvent conditions.

In this paper, we report the results of high field (500 MHz) proton NMR investigation on [Leu[5]]- and [Met[5]]-enkephalinamide acetates in DMSO-d_6. The proton-proton vicinal coupling constants, temperature coefficient of the amide protons and NOE effect in 2-D mode have been utilised to probe the conformational behaviour of these molecules in solution. The effect of amidation on the conformation of native pentapeptides and the conformation-activity relationship are discussed.

Materials and methods

Leucine and methionine enkephalinamide acetates were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. The compounds (2·5 mg) were dissolved in 0·5 ml of 99·8% DMSO-d_6. The solvent used had a trace of water and the signal arising from it has been identified.

One and two-dimensional proton magnetic resonance measurements were carried out on an AM-500 Bruker FT-NMR spectrometer. The temperature of the sample was maintained at 300 K. The resonance peak of DMSO was used as internal reference and the chemical shifts were converted relative to sodium 3-(trimethylsilyl) propionate (TSP) by adding 2·6 ppm to the observed shift values. ^{1}H spectra of [Leu[5]]- and [Met[5]]-enkephalinamides are shown in figure 1. ^{1}H spectra were also recorded as a function of temperature (range 300–360 K).

Two-dimensional correlated spectroscopy (COSY) and NOE effect spectroscopy (NOESY) experiments were carried out with data matrices of 512×2048 and 256×1024 respectively and 64 transients. Mixing time of 600 ms. was used for the NOSEY experiment. The time domain data were multiplied by appropriate window functions before Fourier transformation. J-resolved spectrum was recorded with a data matrix of 128×4096.

Results

Figure 1. 500 MHz ¹H-NMR spectra of leucine (a) and methionine (b) enkephalinamide in DMSO-d_6 at 300 K. The assignment of resonances is indicated in the figure and the shifts are relative to TSP.

residues were identified by 2-dimensional COSY (figure 2) (Aue *et al.*, 1976). These assignments are in conformity with those reported earlier for [Met⁵]- and [Leu⁵]-enkephalins (Jones *et al.*, 1976; Garbay-Jaureguiberry *et al.*, 1977). The methyl resonances from CH_3COO^- and CH_3-S of [Met⁵]-enkephalinamide were assigned on the basis that CH_3COO^- usually resonates around 2·2 ppm. The coupling constant data from J-resolved spectrum along with homonuclear decoupled spectra were used to estimate the proton-proton coupling constants and the chemical shifts of various protons. These NMR parameters are given in tables 1 and 2.
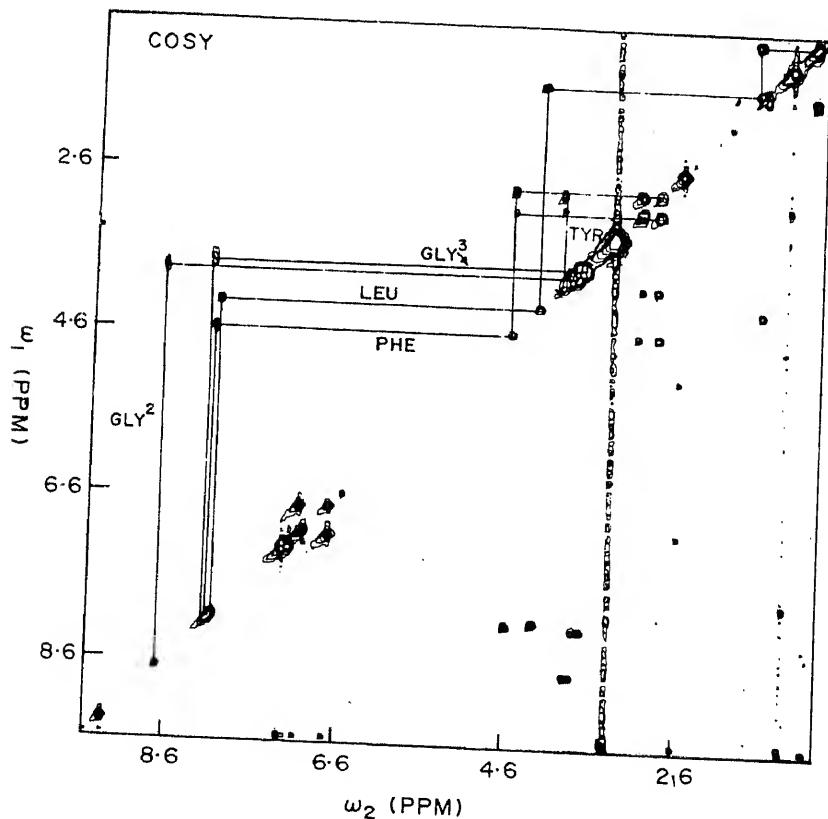
Figure 2. 2D-COSY spectrum of leucine enkephalinamide recorded with 512 × 2048 data matrix size. The digital resolution is 15·6 Hz.

The hydroxyl proton of tyrosine was identified as a sharp singlet at 9·55 ppm in [Leu⁵]-enkephalinamide and as a relatively broad peak at 9·37 ppm in [Met⁵]-enkephalinamide. The resonances from the acetate group CH_3COO^- were identified at 1·25 and 2·12 ppm in the spectra of [Leu⁵]- and [Met⁵]-enkephalinamide respectively. The

Table 1. Proton chemical shifts* in leucine and methionine enkephalinamide (acetate salt) in DMSO-d_6.

Residue	CH_3COO^-	OH	$\overset{O}{\overset{\|}{C}}$-$NH_2$	NH	$C^\alpha H$	$C^\beta H_I$	$C^\beta H_{II}$	$C^\gamma H_I$	$C^\gamma H_{II}$	$C^\delta H_3$	$C^\epsilon H_3$	o	m	p
Leucine enkephalinamide														
Tyr¹	1·25	9·55	—	—	4·05	3·28	3·08	—	—	—	—	6·81	7·16	—
Gly²	—	—	—	8·80	3·93	—	—	—	—	—	—	—	—	—
Gly³		—	—	8·24	3·82 / 3·72	—	—	—	—	—	—	—	—	—
Phe⁴		—	—	8·21	4·63	3·30	3·05	—	—	—	—	7·28	7·28	7·25
Leu⁵		—	7·23 / 7·04	8·15	4·30	1·55	1·55	1·63	—	0·99 / 0·93	—	—	—	—
Methionine enkephalinamide														
Tyr¹	2·12	9·37	—	—	3·48	2·98	2·59	—	—	—	—	6·76	7·06	—
Gly²	—	—	—	8·30	3·76	—	—	—	—	—	—	—	—	—
Gly³	—	—	—	8·18	3·78 / 3·70	—	—	—	—	—	—	—	—	—
Phe⁴	—	—	—	8·20	4·58	3·15	2·92	—	—	—	—	7·29	7·29	7·26
Met⁵	—	—	7·15	8·12	4·33	1·94	2·06	2·55	2·48	—	2·0	—	—	—

*Shifts are downfield relative to TSP.

Table 2. Proton-proton coupling constants in leucine and methionine enkephalinamide (acetate salt).

Residue	$^3J_{NH-C^\alpha H}$	$^2J_{\alpha\alpha}$	$^2J_{\beta\beta}$	$^3J_{C^\alpha H-C^\beta H_I}$	$^3J_{C^\alpha H-C^\beta H_{II}}$	$^3J_{C^\beta H_I-C^\gamma H}$	$^3J_{C^\beta H_{II}-C^\gamma H}$	$^3J_{C^\gamma H-C^\delta H_3}$	$^3J_{om}$
Leucine enkephalinamide									
Tyr[1]	—	—	−14·3	8·3	4·8	—	—	—	8·6
Gly[2]	5·4	−17·0	—	—	—	—	—	—	—
Gly[3]	6·0	−17·0	—	—	—	—	—	—	—
Phe[4]	8·6	—	−13·7	9·7	4·6	—	—	—	—
Leu[5]	8·3	—	−13·7	7·3	7·3	7·0	7·0	6·8	—
Methionine enkephalinamide									
Tyr[1]	—	—	−13·8	9·0	4·5	—	—	—	8·5
Gly[2]	—	—	—	—	—	—	—	—	—
Gly[3]	5·8	−16·5	—	—	—	—	—	—	—
Phe[4]	8·0	—	−14·0	9·8	4·6	—	—	—	—
Met[5]	8·1	—	−13·3	8·9	5·0	*	*	—	—

*Not obtained.

he chemical shifts of the amide, carboxyamide and hydroxyl protons in [Leu^5]- and Met^5]-enkephalinamides were monitored in the temperature range of 300–360 K. l these protons are observed to shifts upfield with increasing temperature. The mperature coefficients dδ/dt observed for the amide, hydroxyl and carboxyamide otons are given in table 3. The amide proton assigned to the Gly^2 starts changing with H_2O beyond 340 K in [Met^5]-enkephalinamide while it does not change in [Leu^5]-enkephalinamide in the same temperature range. The rboxyamide protons in [Leu^5]-enkephalinamide are non-equivalent and their sonances probably collapse to a single resonance near 360 K. The carboxyamide otons of [Met^5]-enkephalinamide are equivalent at room temperature and exhibit sharp resonance which becomes relatively broader at higher temperatures. The rosine hydroxyl protons in [Leu^5]- and [Met^5]-enkephalinamides have similar mperature coefficients (table 3).

Table 3. Temperature coefficient of amide, hydroxy and carboxyamide protons and side chain rotameter populations in [Leu^5]- and [Met^5]-enkephalinamides.

Residue	Temperature coefficient $\frac{d\delta}{dt}(\times 10^{-3}$ ppm/°C)				Rotamer populations		
	NH	OH	NH^{cis}	NH^{Trans}	g^-g^+	tg^+	tg^-
Leucine enkephalinamide							
Tyr^1	—	−5·1	—	—	0·28	0·52	0·20
Gly^2	−4·7	—	—	—			
Gly^3	−4·6	—	—	—			
Phe^4	−4·6	—	—	—	0·17	0·65	0·18
Leu^5	−5·5	—	−5·6	−5·6			
Methionine enkephalinamide							
Tyr^1	—	−6·3	—	—	0·25	0·58	0·17
Gly^2	−5·0	—	—	—			
Gly^3	−5·6	—	—	—			
Phe^4	−5·6	—	—	—	0·17	0·65	0·18
Met^5	−5·7	—	−5·8	−5·8			

iscussion

he temperature coefficient values of the amide protons of various amino acid sidues in [Leu^5]- and [Met^5]-enkephalinamides suggest that none of the amide otons is involved in intramolecular hydrogen bonding. This means that all the nide protons are exposed to the solvent and the observed temperature variation is e to the breaking of intermolecular hydrogen bonds with solvent molecules on creasing temperature. In the absence of any evidence for intramolecular hydrogen

conditions of solvent and temperature (Jones *et al.*, 1977; Higashijima *et al.*
The folded conformation of the native peptides is due to the electrostatic inte
between the positively charged $-NH_3^+$ and negatively charged $-COO^-$ gro
the terminii of the molecule (Higashijima *et al.*, 1979). This interaction is, h
absent in the carboxyamide derivatives which have been investigated here.

The observation of NOESY cross peaks reflects the spatial disposition of
hydrogen atoms and thus provides insight into the 3-dimensional structure
molecule in solution (Jenner *et al.*, 1979; Kumar *et al.*, 1980). The observa
NOESY cross peaks between N_iH and $N_{i+1}H$ and $C_i^\beta H$ and $N_{i+1}H$ reflects lc
short range ordering in the molecule. From the NOESY spectra shown in fi
and 4, it is quite clear that there is no long or short range ordering i
molecules. NOESY cross peaks between $C_i^\alpha H$ and $N_{i+1}H$ for some of the ami
residues have been observed (figures 3 and 4) and these indicate the prefere
extended backbone in both molecules. This deduction is in agreement with
crystallographic data: [Leu^5]-enkephalin has 4 molecules per unit cell and a
molecules have extended backbone conformation (Karle *et al.*, 1983).
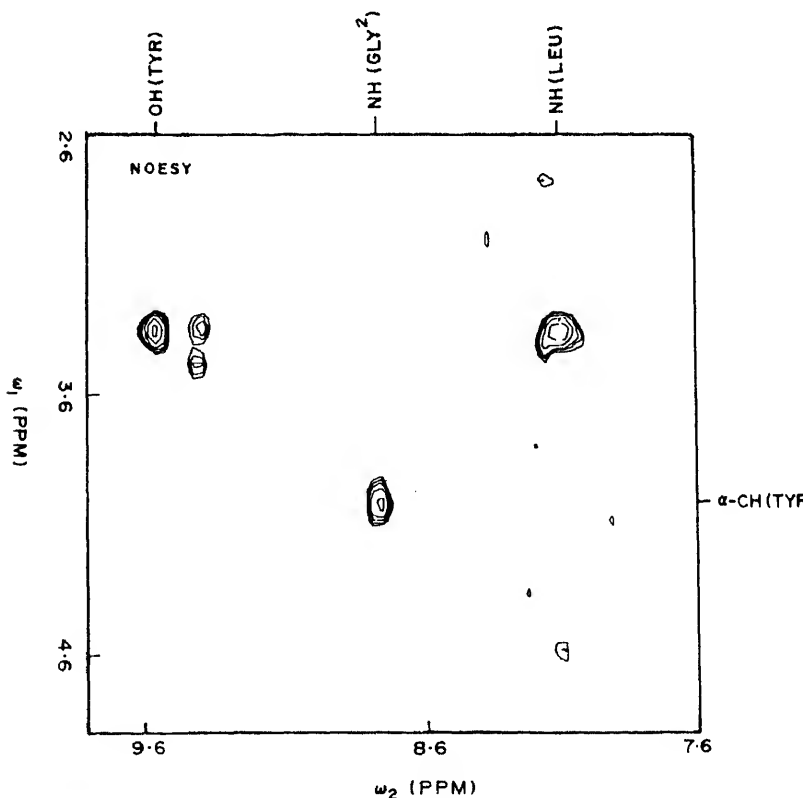
Figure 2. 2D NOESY spectrum of leucine enkephalin amide with mixing time
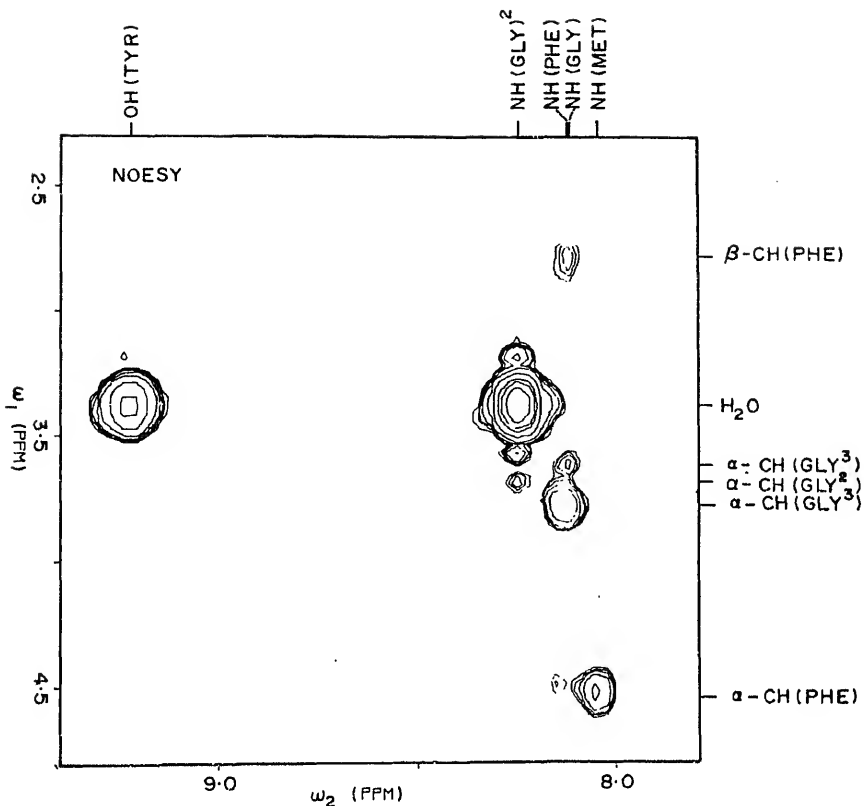
Figure 4. 2D-NOESY spectrum of methionine enkephalinamide with mixing time of 600 ms and data matrix of 256 × 1024.

The most significant difference between the two spectra is the down field shift of H of tyrosine in [Leu⁵]-enkephalinamide (≈ 0·5 ppm) relative to the corresponding ton in [Met⁵]-enkephalinamide.

Methylene protons of Gly² in [Met⁵]-enkephalinamide are equivalent while the responding protons in [Leu⁵]-enkephalinamide are non-equivalent and have ɔstantial shift differences.

Methyl protons of CH_3COO^- ion in [Leu⁵]-enkephalinamide are shifted very ɔch upfield relative to the corresponding protons in [Met⁵]-enkephalinamide. The ignitude of the shift difference will increase further if the assignment of CH_3 onances from CH_3COO^- and CH_3-S of methionine are interchanged.

The −NH proton of Gly² in [Met⁵]-enkephalinamide starts exchanging with O at 340 K while the corresponding proton in [Leu⁵]-enkephalinamide is ɔervable even upto 360 K. The former resonates at a higher field than the latter.

The carboxyamide protons in [Met⁵]-enkephalinamide are equivalent while ɔse in [Leu⁵]-enkephalinamide are non-equivalent and the shift difference between

(vii) The shift difference between β-CH_2 protons of tyrosine in [Met^5]-enkephalin-amide is much larger than that in [Leu^5]-enkephalinamide.

The shift variation of $\simeq 0.5$ ppm in amino acid residues is usually observed for $C^\alpha H$ proton whenever there is a protonation/deprotonation taking place at the $-NH_2$ group (James, 1975). The observed difference of $\simeq 0.5$ ppm between the $C^\alpha H$ protons of [Leu^5]- and [Met^5]-enkephalinamide indicates that the terminal $-NH_2$ group is protonated in [Leu^5]-enkephalinamide while it is not in [Met^5]-enkephali-namide. However, a comparison of the intensity of methyl resonance from CH_3COO^- in both analogues with the intensities of CH_3S (1:1) and $\delta-CH_3$ (1:2) resonances in [Met^5]- and [Leu^5]-enkephalinamides, respectively, clearly establishes the 1:1 stoichiometry and confirms the nature of these amides as acetate salts. Such a large shift differential is difficult to explain unless there are drastic local conformational differences between the two analogues. The existence of conformational differences is supported by the following observations also.

The non-equivalence of methylene protons of both Gly^2 and Gly^3 in [Leu^5]-enkephalinamide clearly indicates some kind of order in this region of the molecule. The same region is however relatively more flexible in [Met^5]-enkephalinamide, where the protons of the CH_2 group of Gly^2 are equivalent while those of Gly^3 are non-equivalent.

The unusually large upfield shift of CH_3 resonance of CH_3COO^- in [Leu^5]-enkephalinamide can be explained only if it is assumed that the time average geometry of the molecule is such that CH_3COO^- is sandwiched between the two aromatic rings of the molecule and the ring current effects will shift CH_3 resonance to high field. This conformation is similar to one of the 4 conformations of [Leu^5]-enkephalin observed in X-ray diffraction studies (Karle et al., 1983).

The differences in the NOESY spectra (figures 3 and 4) obtained under similar experimental conditions (temperature, mixing time, etc.) also indicate conformational differences: the larger number of NOESY cross peaks observed between $N_{i+1}H$ and $C_i^\alpha H$ in [Met^5]-enkephalinamide than in [Leu^5]-enkephalinamide indicates that the backbone is relatively more rigid in the former than in the latter. However, the upfield shift observed for the methyl peak of CH_3COO^- in [Leu^5]-enkephalinamide reflects a concerted motion of the rigid side chains of Tyr and Phe and the ordered backbone. This motion is relatively less concerted in the [Met^5]-analogue.

At neutral pH in DMSO-d_6 the native peptides prefer a folded conformation (Garbay-Jaureguiberry et al., 1976, Jones et al., 1976) while the amides prefer an extended backbone geometry. This change is due to the absence of electrostatic interaction in the amides; the same interaction, due to positive and negative charges on the terminii, is responsible for the folding of the chain in the native peptides (Higashijima et al., 1979). Since the amides are relatively more potent than the native peptides, it can be concluded from the present studies that the requirement of a folded conformation may not be essential and it is probably the local conformation in the backbone and the nature of functional groups that are more critical for interaction of the enkephalins with their receptor than the overall conformation of the molecule.

solution. Secondly, the backbone in [Met⁵]-enkephalinamide is relatively more rigid
than in [Leu⁵]-enkephalinamide. Thirdly, the side chains particularly of Tyr and Phe
in both the amides have definite preferences. Finally, the characteristic folded β-bend
conformation of the endogenous peptides, *i.e.*, [Leu⁵]- and [Met⁵]-enkephalins is
not retained when the C-terminal end is altered. The replacement of $-COOH$ group
by $-CONH_2$ group leads to an extended backbone structure in these pentapeptides.
Since the amides are relatively more potent than the native peptides, the
characteristic β-bend conformation of the native peptide is not essential for their
activity.

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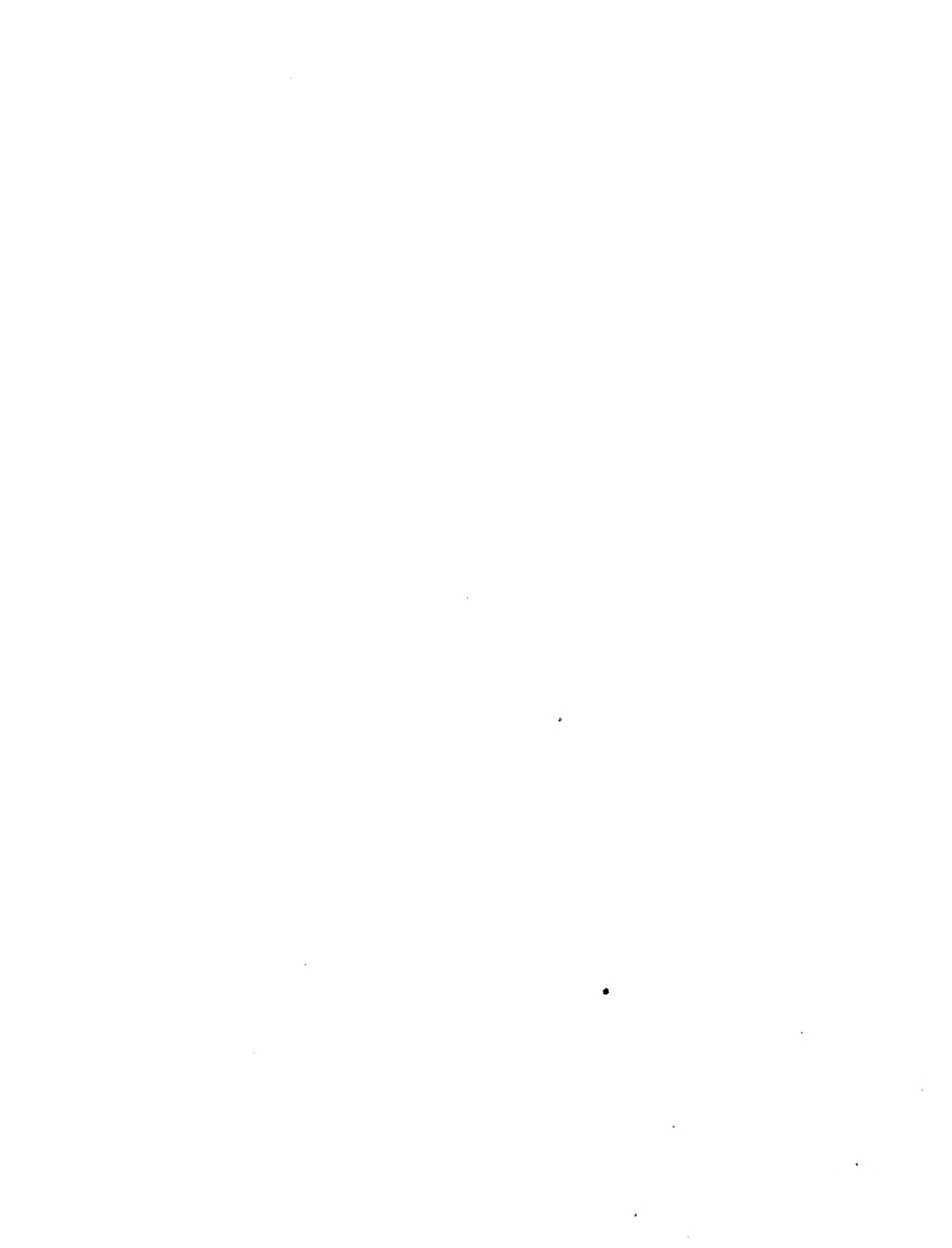
thesis of ribulose 1, 5—bisphosphate carboxylase by isolated *Sorghum* sophyll chloroplasts

T. VALLIAMMAI†, S. KRISHNASAMY and A. GNANAM*

Department of Plant Sciences, School of Biological Sciences, Madurai Kamaraj University, Madurai 625 021, India

*Present address: Vice chancellor, Bharathidasan University, Tiruchirapalli 620 024, India

Abstract. Chloroplasts isolated from *Sorghum vulgare* are active in light-dependent, organelle protein synthesis. Intact chloroplasts can use light as an energy source; photosynthetically inactive chloroplasts require the addition of ATP for this protein synthesis. Preincubation of chloroplasts in light at 25°C for 1 h depleted the endogenous templates completely; such preincubated chloroplasts translated exogenously added heterologous templates efficiently. When total cellular RNA from *Chlorella protothecoides*, a C₃ plant, was used as template for translation in a cell-free light-dependent system of isolated mesophyll chloroplasts from *Sorghum vulgare*, a C₄ type plant, polypeptides of 55 kDa (large subunit) and 15 kDa (small subunit) were detectable in the fluorographic profile of the newly synthesized proteins; these polypeptides were absent in the products obtained with endogenous RNA. Evidence for the fidelity of the system was obtained by immunological analysis of ribulose 1, 5-bisphosphate carboxylase obtained by the translation of *Chlorella* cellular RNAs.

Keywords. *Sorghum* mesophyll chloroplasts; *in vitro* translation; product analysis.

oduction

earch on the biogenesis of chloroplasts has gained much impetus in recent years studies on organelle protein synthesis using isolated chloroplasts. This approach vides the most direct information on which proteins are made on chloroplast somes. Isolated chloroplasts from a number of plant species can perform light-en protein synthesis *in vitro* (Ramirez *et al.*, 1968; Blair and Ellis, 1973; omley *et al.*, 1974; Mendiola Morgenthaler *et al.*, 1976; Vasconcelos, 1976; Geetha Gnanam, 1980; Geetha *et al.*, 1980; Ellis, 1981; Colijn *et al.*, 1982; Leu *et al.*,). Up to 90 discrete polypeptide species can be synthesized with this system s, 1977, 1981). Therefore the isolated system represents a powerful tool for ying the expression of the chloroplast genome.

ibulose 1,5-bisphosphate (RuBP) carboxylase the primary carboxylating enzyme ₃ plants is a large multimeric protein (above $5–5\cdot5 \times 10^5$ daltons) found in the ma fraction of chloroplasts (Kawashima and Wildman, 1970; McFadden, 1973). enzyme is composed of multiple copies of two non-identical subunits designated subunit (LSU) with a relative molecular mass (M_r) of 50–60 kDa, and small subunit) of 12–20 kDa (Sugiyama *et al.*, 1971; Givan and Criddle, 1972; Gray and wick, 1974; Iwanij *et al.*, 1974). It is now well established that the LSU is coded by the chloroplast genome and its mRNAs are translated on chloroplast ribo-

cytoplasmic ribosomes as a precursor and transported into the chloroplasts (Cl

et al., 1970; Blair and Ellis, 1973; Kung, 1976). RNAs extracted from spinach

Euglena chloroplasts have been used to synthesize the LSU in heterologous t

lational systems derived from Escherichia coli or wheat germ (Hartely et al.,

Sagher et al., 1976). RNA from whole cell extracts of Chlamydomonas reinhardti

also shown to be translated into the LSU in an E. coli ribosomal system (He

et al., 1977).

So far all the studies on in vitro chloroplast protein synthesis have been con

entirely to the characterization of products formed on endogenous temp

However, depletion of the endogenous templates by preincubation of the ch

plasts in light (Geetha and Gnanam, 1980; Geetha et al., 1980) permitted us to

chloroplasts as a cell-free assay system to study the products formed with a

RNA templates. The system is similar to the S-30 fraction of E. coli (Nirenberg

Matthaei, 1961), wheat germ (Robertz and Paterson, 1973), or rabbit reticulo

(Lodish and Desalu, 1973). In this paper we communicate data from our attemp

translate RuBP carboxylase from C_3 whole cell RNAs in preincubated meso

chloroplasts of Sorghum vulgare.

Materials and methods

Chloroplast isolation

Chloroplast from S. vulgare were isolated by the method of Geetha and Gna

(1980). Five g of young leaves of 8–12 day-old field-grown plants were illumin

under photoflood lamps of 20,000 lux for 15 min after surface sterilization with

sodium hypochlorite. The leaves were then homogenized twice for 5 s at 50%

voltage in a Sorvall Omni Mixer in 50 ml of partially frozen sterile isolation me

containing 50 mM Tris-HCl buffer pH 8·5, 330 mM sorbitol, 4 mM $MgCl_2$

2 mM β-mercaptoethanol. The homogenate was rapidly squeezed through 8 lay

muslin and centrifuged at 2,500 g for 1 min. The supernatant was decanted an

pellet was carefully resuspended in the same medium. The entire procedu

isolation of chloroplasts from the homogenisation step to final resuspension

done within 2 min at 4°C. Chlorophyll was estimated according to Arnon (194

Assay for protein synthesis

For light-driven protein synthesis, 0·1 ml of chloroplast suspension (5–15 μg ch

phyll) was added to 0·5 μCi of [^{14}C]-labelled algal hydrolysate or [^{35}S]-methi

at 4°C. The reaction was started by increasing the temperature of the incub

mixture to 25°C in a water bath illuminated with photoflood lamps of 20,00

appropriate dark controls were maintained simultaneously. After desired tim

incubation, known aliquots were transferred to Whatman No. 3 filter discs and

quickly with a hot-air blower. The discs were immediately immersed in 10%

trichloroacetic acid (TCA) and processed by the method of Bollum (1960) as desc

by Mans and Novelli (1961). The processed filter paper discs were air drie

radioactivity was measured using a Packard liquid scintillation counter. To min

opriate amount of *Chlorella* RNA in a total volume of 100 μl. Appropriate dark
light controls of reaction mixture containing preincubated chloroplasts without
d templates were maintained.

tal cellular RNA from *Chlorella protothecoides* was extracted by the modified
phenol method (Girard, 1967). In *C. protothecoides*, maximum RNA synthesis
rred around 20 h of greening and RNA used for *in vitro* translation studies were
ined at this stage of development (Aoki and Hase, 1964).

fication of RuBP carboxylase from Vigna sinensis

P carboxylase was purified according to the method of Kawashima and
man (1971) with some modifications. Antisera were prepared by immunizing
its with the purified RuBP carboxylase diluted to 2 mg/ml in 0·025 M Tris-HCl
r pH 7·4.

um dodecyl sulphate-polyacrylamide gel electrophoresis and fluorography

ein was estimated according to Lowry *et al.* (1951). Sodium dodecyl sulphate
)-polyacrylamide gel electrophoresis (PAGE) was carried out as described by
nmli (1970) with some modifications. Polyacrylamide gradient gels (7·5–15%)
used. The stacking gel contained 4% acrylamide. Electrophoresis was carried
at 25°C for 12 h at 20 mA with the initial 1 h at 10 mA. Electrophoresis was
ped after the marker dye reached the bottom of the gel. The stained gel was used
uorography (Bonner and Laskey, 1974). The developed X-ray film was scanned
g MD-100 microdensitometer.

ie gel was calibrated using the following molecular weight standards: bovine
m albumin, 66,000; egg albumin, 45,000; β-lactoglobulin, 18,400 and lysozyme,
)0.

aration of immunoprecipitates

immunoprecipitation of RuBP carboxylase was carried out following the
edure of Ross and Schatz (1976) with some modification. One hundred μl of
M sodium phosphate, pH 7·4, were added to 100 μl of chloroplast suspension.
Triton X-100 was added (to 1%, v/v) to this suspension to solubilize the enzymes.
suspension was mixed thoroughly and kept at room temperature for 10 min with
sional mixing. It was centrifuged at 35,000 g for 10 min. The supernatant was
ed 10 times with TNET buffer (50 mM Tris-HCl pH 7·4, 0·15 M NaCl, 5 mM
A and 1% Triton X-100). Ten μl of purified RuBP carboxylase were added as a
er protein. Twenty μl of antiserum in 0·1 ml of buffer were then added and the
ure was incubated overnight at 4°C with occassional shaking. Forty μl of a 10%
) suspension of fixed *S. aureus* cells were added, the suspension kept at room

was removed and the pellet was washed with TNET buffer thrice. The pellet was then dissolved in 1% SDS and the resulting suspension was centrifuged at 35,000 g for 15 min. The supernatant was used for the determination of radioactivity and SDS-PAGE.

Results

Light-driven protein synthesis

A time course study of the incorporation of [^{14}C]-labelled amino acids into hot TCA-insoluble material by isolated *Sorghum* mesophyll chloroplasts is shown in figure 1. The incorporation of amino acids into polypeptides was light-dependent. However, light could be substituted by added ATP in the dark. Amino acid incorporation was linear only for 20 min and levelled off thereafter.
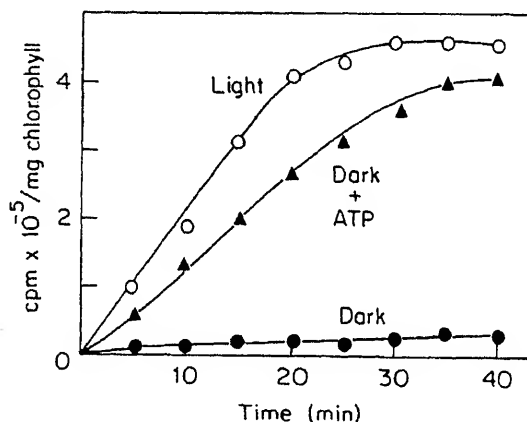
Figure 1. Time course of [^{14}C]-labelled amino acids incorporation into polypeptides by isolated mesophyll chloroplasts of *S. vulgare*. Light; ($\triangle$), dark + 5 mM ATP; ($\bullet$), dark.

Light-driven protein synthesis with exogenous templates

Having established that protein synthesis in isolated chloroplasts ceases within 25 min, attempts were made to find out the possible reason for this short lived synthetic activity. One possibility is that due to the absence of an active transcriptional process, protein synthesis stops as soon as the already existing endogenous mRNAs are depleted. This possibility was tested adding RNA obtained from actively metabolising cells of *C. protothecoides*. In this experiment one aliquot of chloroplast preparation was incubated with [^{14}C]-labelled amino acids under light (fresh chloroplasts). Another aliquot was incubated under light at 25°C for 1 h without any [^{14}C]-labelled amino acids (preincubated chloroplasts). During the preincubation the endogenous mRNAs would have been depleted and very little incorporation of
labelled amino acids occurred after this period. When these chloroplasts were

Table 1. Restoration of protein synthetic activity of the pre-incubated *Sorghum* mesophyll chloroplasts (depleted of their endogenous templates) by the addition of exogenous templates.

Nature of the chloroplast and the assay conditions	Protein synthesized [CPM × 10⁻⁵]	
Fresh chloroplasts		
Dark	96,889	0·9
Light	691,828	6·9
Pre-incubated chloroplasts		
Light (without exogenous template)	81,789	0·8
Dark + 50 μg of *Chlorella* RNA	97,532	0·9
Light + 50 μg of *Chlorella* RNA	801,556	8

Total cellular RNA obtained from the *Chlorella* cells served as the exogenous template (50 μg RNA/100 μl reaction mixture). The conditions for the protein synthesis were as described under materials and methods.

The dose response curve for the protein synthetic activity by preincubated chloroplasts with the added RNA from *Chlorella* cells is shown in figure 2. Maximum protein synthetic activity by the preincubated chloroplasts was obtained with 50 μg of exogenous RNAs in a final reaction mixture of 100 μl.
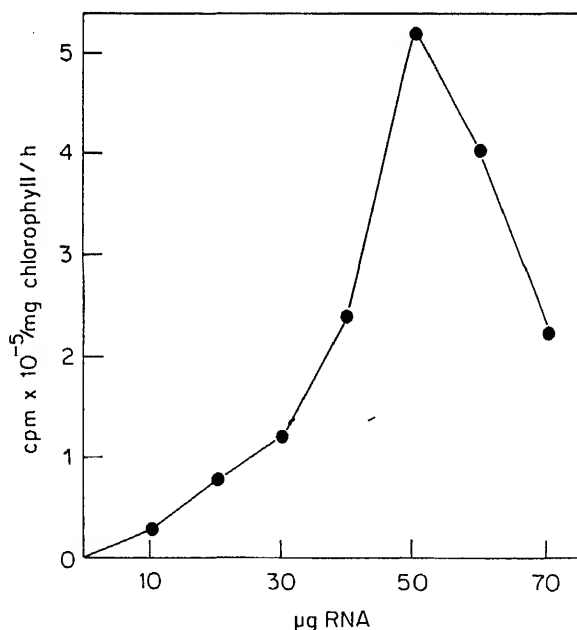
Figure 2. Effect of concentration of added RNAs on protein synthetic activity by preincubated chloroplasts of *S. vulgare*.
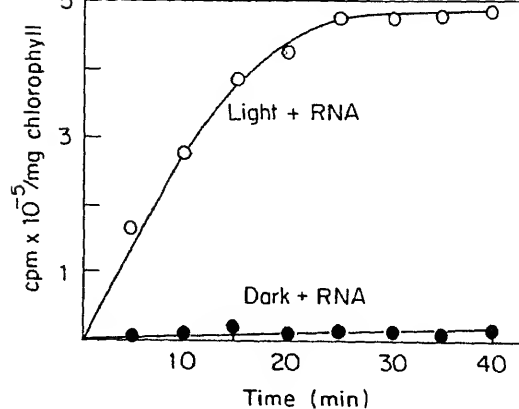
Figure 3. Time course of $[^{14}C]$-labelled amino acid incorporation *in vitro* into polyp[...]
by *S. vulgare* chloroplasts provided with *Chlorella* total RNA. (○), Light + 50 μg RN[...]
dark + 50 μg RNA.

dependent on light. Almost no activity was observed in dark incubated chloro[...]
incubated with *Chlorella* RNA.

Figure 4 shows the SDS-PAGE analysis of products of protein synthes[...]
isolated *Sorghum* chloroplasts with endogenous (lane a) and exogenous (la[...]
RNAs. There was synthesis of more than 15 different polypeptides with M_r of 7[...]
69, 67, 65, 60, 57, 50, 48, 46, 43, 41, 40, 34, 32, 20, 18 and 15 kDa (figure 4C, la[...]
and b), both in fresh chloroplasts making use of endogenous templates and i[...]
incubated chloroplasts with exogenous RNAs. The Coomassie blue stained[...]
(figure 4A) and the fluorographic profiles (figure 4B) of the products of pr[...]
synthesis with endogenous (lane a) and exogenous (lane b) RNAs were qualita[...]
similar but for the presence of a polypeptide with M_r of 55 kDa in the protein p[...]
obtained with exogenous RNAs. This polypeptide of 55 kDa (figure 4B,C lane[...]
comparable to the LSU of RuBP carboxylase. Figure 4C shows the relative ch[...]
in the quantities of various polypeptides in the fluorographic profiles of produ[...]
protein synthesis with endogenous and exogenous RNAs. In preincubated cl[...]
plasts with exogenous templates, the polypeptides with M_r of 60, 41, 34, 32, 2[...]
15 kDa were found to be translated more than in fresh chloroplasts with endog[...]
templates.

Immunological identification of RuBP carboxylase

Using antiserum against RuBP carboxylase, attempts were made to detec[...]
determine the relative amount of RuBP carboxylase in the products of p[...]
synthesis by isolated *Sorghum* mesophyll chloroplasts using either endog[...]
mRNA or added total RNA from *C. protothecoides*. In these experiments the[...]
ducts of protein synthesis were subjected to immunoprecipitation with anti[...]
against RuBP carboxylase. An appreciable amount of radioactivity was found[...]
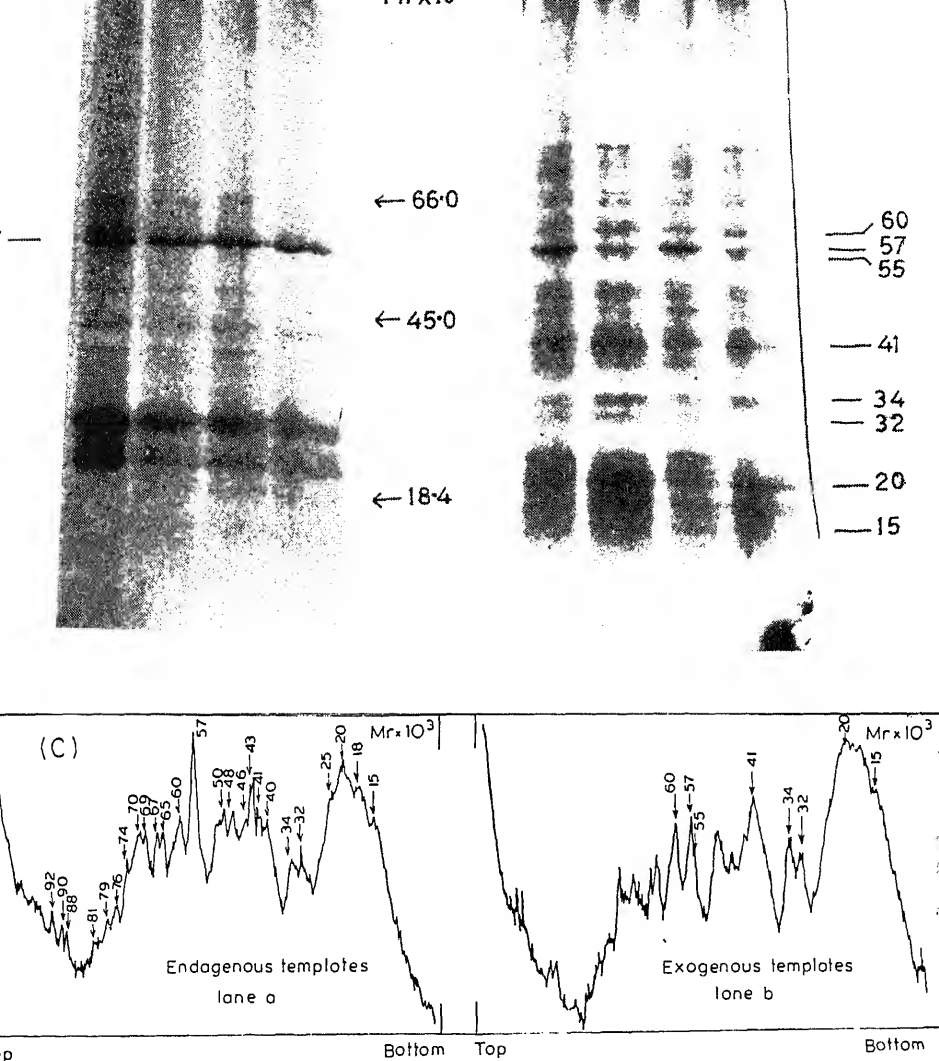
Figure 4. SDS-PAGE pattern of [³⁵S]-methionine labelled products of *in vitro* protein synthesis by isolated *S. vulgare* mesophyll chloroplasts. Equal amounts of protein (150 μg) were loaded. The arrows indicate marker proteins. A. Coomassie blue stained gel. B. Fluorography of the same gel. C. Densitometric tracings of the fluorograph. Lanes a and b shown in B were scanned in an MD-100 micro densitometer. Lane a, endogenous templates; b, exogenous templates (*Chlorella* RNA).

Analysis of the immunoprecipitates by SDS-PAGE revealed that LSU (55 kDa) and SSU (15 kDa) of RuBP carboxylase are synthesized in preincubated chloroplasts using exogenous template (*Chlorella* RNA, figure 5, lane b) but not in chloroplasts

Table 2. Radioactivity associated with the immunoprecipitate formed between antiserum of native RuBP-carboxylase and products of *in vitro* translation with the template of different origin. The table shows the results of 3 different experiments.

Nature of the template used	Radioactivity associated with immunoprecipitate CPM		
	I	II	III
Endogenous template	79	114	108
Total cellular RNA from *Chlorella* (50 μg/100 μl reaction mixture)	2180	1785	2432

The immunoprecipitates of products translated *in vitro* were allowed to bind with the fixed *Staphylococcus aureus* cells. After the centrifugation the cells were separated from antigen-antibody complex by adding 1% SDS. The supernatant obtained after the centrifugation was used for counting the radioactivity. The presence of radioactivity in this supernatant was indicative of synthesis of RuBP carboxylase by the isolated chloroplasts.

Discussion

Isolated *Sorghum* mesophyll chloroplasts could carry out protein synthetic activity using light as the only source of energy as in the case with chloroplast preparations from other species of plants (Ramirez *et al.*, 1968; Blair and Ellis, 1973; Bottomley *et al.*, 1974; Mendiola Morgenthaler *et al.*, 1976; Vasconcelos, 1976; Geetha and Gnanam, 1980; Geetha *et al.*, 1980; Colijn *et al.*, 1982; Leu *et al.*, 1984). However, light could be substituted by added ATP in the dark. Blair and Ellis (1973) and Bottomley *et al.* (1974) have shown that isolated chloroplasts can also use added ATP for protein synthesis when their outer membranes are made permeable by osmotic shock. This conclusion is contrary to our observation that chloroplasts not given osmotic shock carried out protein synthesis with ATP in the dark. The decrease in protein synthetic activity after preincubation could be restored by adding exogenous RNA (table 1) (Geetha and Gnanam, 1980; Geetha *et al.*, 1980). Apparently the endogenous templates are labile and degraded on a time scale equal or similar to the time of preincubation. Regarding the question of the entry of added RNA into chloroplasts, no definitive mechanism can be established. Phase-contrast microscopic observations of the *Sorghum* mesophyll chloroplast preparations used for the *in vitro* translational studies indicated that they contained a mixture of both intact and stripped chloroplasts (Geetha and Gnanam, 1980). It could be that the photophosphorylation coupled protein synthesis occurs only in the intact chloroplasts and the added RNA gains entry into the chloroplasts. Swanson (1971) observed the penetration of RNA species into isolated mitochondria and their involvement in protein synthesis. Macromolecules of cytoplasmic origin which play an important role in chloroplast development are known to be transported across the double membrane into the chloroplasts although the mechanism of this transport and its specificity remain obscure (Hoober *et al.*, 1969; Hoober, 1970; Eytan and Ohad, 1970, 1972;

(A)   (B)

←LSU

←SSU
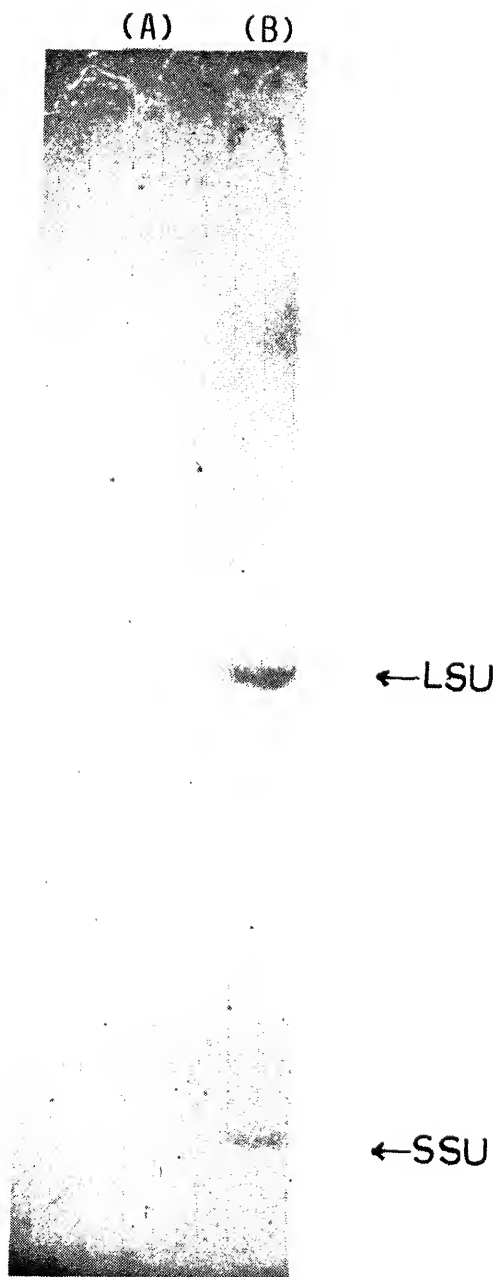
Figure 5. Fluorographic profile of electrophoretically separated immunoprecipitates

reason to believe that intact chloroplasts are needed for photophosphorylation,
broken chloroplast membrane fractions are known to phosphorylate.

Maximum translational efficiency was observed at 50 μg RNA/100 μl of
mixture containing 15 μg chlorophyll equivalent of chloroplasts and supra-op
concentrations of RNA inhibited the protein synthetic activity to a consid
extent (figure 2). The requirement of a critical concentration of RNA has
observed in a number of cell free systems derived from a variety of sources su
wheat-germ (Tobin and Klein, 1975; Bottomley et al., 1976), Krebs ascites tu
(Callis et al., 1975), rabbit reticulocyte (Pelham and Jackson, 1976), Artemia
(Tse and Taylor, 1977) and Sorghum (Geetha and Gnanam, 1980).

One distinguishing feature in the products of protein synthesis by So
mesophyll chloroplasts using endogenous templates was the total absence of
carboxylase. In Sorghum which is a C_4 plant, RuBP carboxylase is found exclu
in the bundle sheath chloroplasts and is almost absent in the mesophyll chloro
(Kanai and Edwards, 1973; Edwards et al., 1974; Ku and Edwards, 1975;
et al., 1976; Kirchanski and Park, 1976; Link et al., 1978) although there are r
stating that in a few graminae members like Digitaria sanguinalis, Dig
decumbens and Cyperus rotunds, 1–6% of the total RuBP carboxylase of lea
located in the mesophyll chloroplasts (Campbell and Black, 1982; Harriso
Black, 1982).

In the case of S. vulgare, the complete absence of RuBP carboxylase fro
mesophyll chloroplasts has been demonstrated by Ouchterlony double immu
fusion technique using anti-native RuBP carboxylase (Geetha et al., 1980). Kirc
and Park (1976), using SDS-PAGE analysis of proteins of mesophyll and b
sheath chloroplasts, clearly showed the localization of RuBP carboxylase exclu
in the bundle sheath chloroplasts. Link et al. (1978) have shown that in Zea m
DNA sequence coding for the LSU of RuBP carboxylase is present in the c
plast DNA of both mesophyll and bundle sheath cells. However, the transl
mRNA for this polypeptide is detectable only in the bundle sheath chloroplas
is entirely absent from mesophyll chloroplasts.

When preincubated Sorghum mesophyll chloroplasts were provided with
cellular RNA from Chlorella, polypeptides with M_r of 55 and 15 kDa were dete
in the fluorographic profile of the newly synthesized proteins, these polype
were absent from the products obtained with endogenous templates (figure
These polypeptides can be equated to the LSU and SSU of RuBP carboxyla
their presence in the in vitro translational products indicates that the mRNA fo
and SSU of RuBP carboxylase were present in intact form in the total cellula
preparation from Chlorella and were effectively translated in the S
chloroplasts. Geetha et al. (1980) have also demonstrated the synthesis of bo
subunits of RuBP carboxylase in Sorghum mesophyll chloroplasts provide
Vigna whole cell RNA. In vitro translation of LSU mRNA in cell-free syste
been demonstrated earlier. Hartley et al. (1975) have shown that the RNA ex
from spinach can direct the synthesis of LSU of RuBP carboxylase in a hetero
translational system derived from E. coli. Sagher et al. (1976) have describ
properties of chloroplast RNA from Euglena which directs the synthesis of L

of Krebs ascites lysate and rabbit reticulocyte ribosomes (Geetha and Gnanam, 1980).

Acknowledgement

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Lipid profile of cultured cells of apple (*Malus sylvestris*) and apple tissue

T. N. PRABHA, P. L. RAINA and M. V. PATWARDHAN

Central Food Technological Research Institute, Mysore 570 013, India

Abstract. The potentiality of apple cell cultures to synthesize not only higher quantities of lipids than apple fruit but also different classes of lipids is noted. Total lipid was 15-fold higher in apple callus than in the original tissue. On callusing, linoleic acid decreased from 66% to 14%, while linolenic acid showed a very large increase from 0·9% to 44%. Stearic and oleic acids also increased in callus. The relative amounts of sterol/hydrocarbon and diglyceride fractions were higher in callus cultures, while apple tissue showed higher levels of tri-glycerides and sterol. Phosphatidylethanolamine and phosphatidylglycerol seemed to be newly synthesized during callusing while other phospholipids such as lysophosphatidyl-choline, lysophosphatidylethanolamine, phosphatidylinositol and phosphatidic acid decreased. There was much higher glycolipid in apple callus than in the original tissue. The ratio of neutral lipid to polar lipid was higher in apple than in apple callus.

Keywords. Apple; callus culture; lipid profile.

Introduction

In recent years, cultured plant cells have received much attention as potential sources of natural products as well as novel cell systems for understanding basic metabolism (Ludden and Carlson, 1980; Staba, 1980). Callus tissue of plant origin has been examined widely for its cytological and morphological characteristics but very little is known about its biochemical nature (Ammirato *et al.*, 1984). Study of biochemistry of plant callus tissue is of recent origin. Mangold (1986) has stressed the importance of understanding the basic nature of cultured cells for obtaining desired metabolites. Lipids are important cellular macromolecules and have been studied to understand the differences between cells of intact tissue and the corresponding cells grown on an artificial medium. Recently, fatty acid composition of callus tissue from cotyledons of 6 species of Cucurbitaceae (Halder and Gadgil, 1983, 1984) was reported. The present study compares lipid composition of apple fruit and apple callus.

Materials and methods

Fully mature fresh 'Golden delicious' apples, *Malus sylvestris*, grown in Kashmir (north India) were used in the experiments. Apple callus was obtained from the edible portion of the fruit by aseptic culture on modified MS medium (Lieberman *et al.*, 1979) containing the following organic supplements; 2,4-dichlorophenoxyacetic acid, 2 ppm; kinetin, 0·3 ppm; thiamine, 10 ppm; niacin, 5 ppm; pyridoxine, 5 ppm; casein hydrolysate, 0·05% and coconut water, 10%. The cultures were maintained at 26°C. Actively growing fluffy callus mass was collected between 45 and 50 days of culturing from the 3rd subculture (130 days after callus initiation).

Extraction and purification of lipid

Lipid extraction, purification and analysis were done according to the proce[dure] described earlier (Mahadevappa and Raina, 1978). Thus, 50–100 g of fresh a[pple] tissue and apple callus were homogenized and extracted thrice in 4 volume[s of] chloroform : methanol (2 : 1 v/v) using α-tocopherol as antioxidant. The chlorof[orm :] methanol layer was collected and concentrated. The lipid was redissolved in chl[oro]form and washed twice with 0·74% aqueous potassium chloride. The chlorof[orm] layer was dried over anhydrous sodium sulphate and finally taken in a kn[own] volume of chloroform.

Neutral and polar lipids were resolved by preparative thin-layer chromatogra[phy] (TLC). Initially, neutral lipids were separated using petroleum ether: solvent e[ther :] acetic acid (80 : 20 : 1) in which polar lipids would remain at the origin. The reso[lved] neutral lipids (excluding the origin) were scrapped off the plate, re-extracte[d in] chloroform : methanol (2 : 1) and rechromatographed individually in the same sol[vent] system. The origin was likewise scraped off, extracted in chloroform : methanol, [and] the lipids thus recovered were chromatographed in chloroform : methanol (4 : [1) to] resolve polar lipids. Individual fractions were scraped off the plate and rechrom[ato]graphed along with authentic standards.

Quantitation of lipid fractions

The TLC plates were lightly sprayed with 50% H_2SO_4, air dried and charre[d to] locate the various lipid components. The relative concentrations of these compon[ents] were determined by the automatic TLC scanner (model 2, mounted on a flourim[eter] model III, Turner associates, California, USA). Identification of the neutral [and] polar lipids on TLC plates was done by comparison with the R_f of the auth[entic] standards and the use of specific spray reagents (Siakotos and Rouser, 1965; L[owry,] 1968, Vaskovsky and Kostetsky, 1968). Sugar (Dubois *et al.*, 1956) and protein (L[owry] *et al.*, 1951) in the lipid were estimated by standard procedures.

Fatty acid methyl esters prepared essentially by the method of Kates (1972) [in] methanolic HCl were analysed by gas chromatography (Packard gas chromatog[raph,] model 237 with flame ionization detector, N_2 flow: 20 ml/min; 7 ft × 1/8″ column [with] 10% w/w DEGS coated on Chromosorb W; 180°C). Unknown peaks were iden[tified] by comparison with authentic standards.

Results

Table 1 indicates significantly increased lipid in apple fruit callus tissue compar[ed to] apple fruit tissue. Polar lipid content of callus was 25 times higher and neutral [lipid] 10 times higher than the corresponding values for fruit. The polar to neutral [lipid] ratio was 1 : 3 in fruit tissue and 1 : 1 in callus cultures.

On callusing, the sugar content of lipid increased from 9·4 to 12 mg and the pr[otein] from 3·9 to 11·6 mg/100 mg lipid. Table 2 shows that there was a considerable ch[ange] in the proportions of fatty acids. Callus had less linoleic acid and more linolenic

Table 1. Lipid content of apple and apple callus.

Lipid type	Apple	Apple callus
	(mg/100 g fresh weight)	
Total lipid	77 ± 4·2	1069 ± 137·0
Neutral lipid	54 ± 4·9	534 ± 29·0
Polar lipid	21 ± 2·9	563 ± 26·7

Each value is the average of 4 replicates.

Table 2. Fatty acid composition of apple and apple callus lipids.

Fatty acid		Total lipids		Polar lipids	
		Apple	Apple callus	Apple	Apple callus
		(%)		(%)	
Lauric	12:0	0·6	0·8	3·0	0·1
Tridecanoic	13:0	—	—	0·2	Tr.
Myristic	14:0	1·3	0·8	0·3	0·4
Myristilic	14:2	0·1	0·3	—	0·1
Pentadecanoic	15:0	—	—	Tr.	0·4
Palmitic	16:0	20·1	22·5	22·2	28·1
Palmitoleic	16:1	0·2	0·4	—	—
Heptadecanoic	17:0	—	—	—	0·2
Stearic	18:0	2·0	4·6	0·9	5·0
Oleic	18:1	6·5	13·0	3·1	12·4
Linoleic	18:2	65·8	13·6	68·2	13·7
Linolenic	18:3	0·9	43·8	0·2	39·3
Arachidic	20:0	1·0	—	0·5	0·3
Unidentified	—	—	1·5	0·2	1·4	—

Thin-layer chromatograms of neutral and polar lipids are shown in figures 1–3. The neutral lipid profiles reveal significantly lower triglyceride fraction and higher sterol ester/hydrocarbon fraction in callus. In addition, diglyceride fraction was higher and sterol fraction lower in callus tissue (table 3).

Table 3. Composition of neutral lipid in apple and apple callus.

Neutral lipid	Apple	Apple callus
	(% Total)	
Origin	0·8	2·2
1. Monoglyceride	2·2	1·8
2. 1:2 diglyceride	2·6	7·2
3. 1:3 diglyceride	1·6	9·0
4. Unidentified	4·8	—
5. Sterol	16·3	12·3
6. Free fatty acid	8·1	6·5
7. Methyl esters of free fatty acids	3·8	1·0
8. Unidentified	3·4	2·2
9. Unidentified	0·8	2·8
10. Triglyceride	30·6	4·0
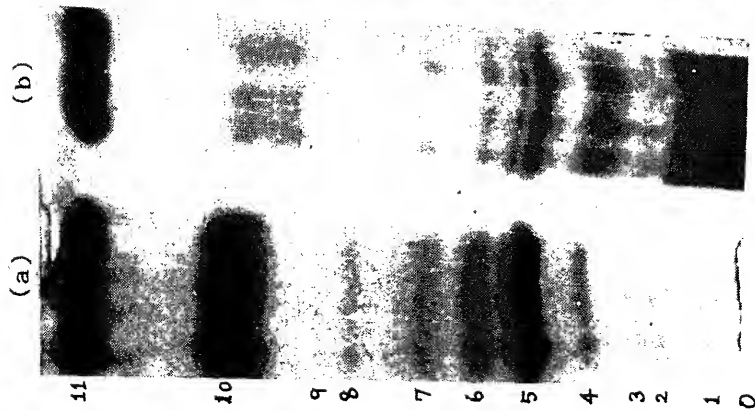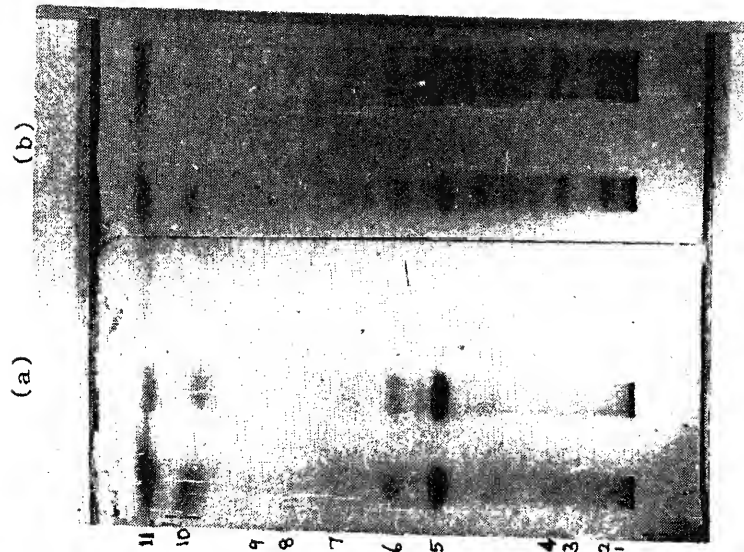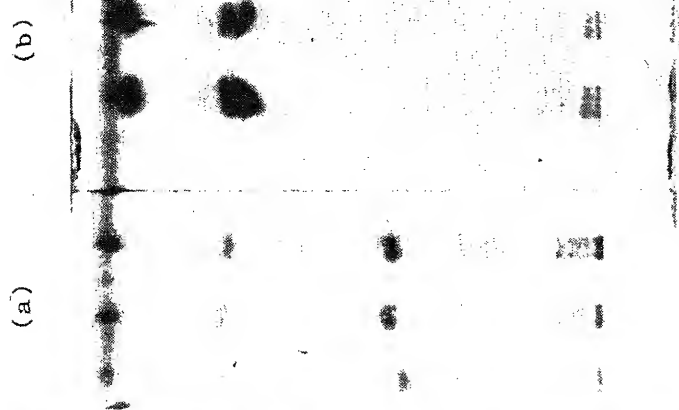
Figures 1–3. 1 and 2. Thin-layer chromatogram of apple (a) and apple callus, (b) neutral lipids. 1. First separation. 2. Rechromatographed exculding the origin from the first separation. 3. Thin-layer chromatogram of apple and apple callus polar lipids, resolved from material remaining at the origin after the first separation of neutral lipids.

Data on polar lipid composition (table 4) reveal a significant difference in the proportions of phosphatidylethanolamine and phosphatidylglycerol. These contributed more than 50% of total polar lipid in apple callus and were present only in traces in apple fruit. Lower relative proportion of other phospholipids in callus, compared to fruit, were also observed. The data also indicate a much higher level of glycolipids (monogalactosyl diglyceride and digalactosyl diglyceride) in callus cells than in fruit. Interestingly, about 2·4% sulphatides were identified in callus; this lipid was not found in fruit.

Table 4. Composition of polar lipid in apple and apple callus.

Polar lipid	Apple	Apple callus
	(% Total)	
Origin	8·6	3·9
1. Lysophosphatidylcholine	13·4	5·5
2. Lysophosphatidylethanolamine	7·3	1·5
3. Sulphatide	—	2·4
4. Unidentified	7·8	Tr.
5. Phosphatidylinositol	9·8	0·5
6. Digalactosyldiglyceride	—	2·6
7. Phosphatidylcholine	23·4	Tr.
8. Unidentified	4·5	0·5
9. Phosphatidylethanolamine	0·4	30·0
10. Monogalactosyldiglyceride	12·0	20·1
11. Unidentified	—	2·5
12. Unidentified	—	0·1
13. Phosphatidylglycerol	—	22·0
14. Phosphatidic acid	12·8	8·4

Lipid numbering scheme is according to mobility in TLC (see figure 3).

Discussion

The reciprocal change in the proportions of linoleic and linolenic acids observed in apple callus was also shown in callus cultures of cotyledons of *Cucumis melo* (Halder and Gadgil, 1983, 1984). Increased glycolipid synthesis was reported in suspension cultures of *Peganum hermala* and *Chenopodium rubrum* (Barz *et al.*, 1980; Huseman *et al.*, 1980). It was also shown that photoautotrophic cultures contained higher amounts of lipids and linolenic acid than heterotrophic cultures (Mangold, 1977, 1980). Large amounts of diacylglycerophosphoethanolamine and diacylglycerophosphocholine and a lower amount of diacylglycerophosphoinositol were observed in heterotrophic cell cultures (Radwan and Mangold, 1976). Similarities in lipid composition between cultured cells and plant tissue have also been demonstrated (Mangold, 1986).

The present study throws some light on the differences in lipid composition between cultured apple cells and apple tissue. It clearly shows that the primary metabolism of cultured cells differs from that of the tissue from which the cultured cells were derived. There was not only an increase in total lipid but also differences in

phospholipids and a change in the ratio of polar to neutral lipids indicate difference in the biosynthesis of lipids during the growth of the tissue.

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Characterization of foot-and-mouth disease virus types O and Asia 1 RNA

S. VASANTHA*†, S. M. LAL* and A. ANTONY§

Microbiology and Cell Biology Laboratory, Indian Institute of Science, Bangalore 560 012, India

*Indian Veterinary Research Institute, Bangalore 560 024, India

†Present address: Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20205, USA

Abstract. Poly(A) RNA was isolated from foot-and-mouth disease virus-infected cells by oligo(dT)-cellulose chromatography. One-dimensional oligonucleotide mapping of virus-induced poly(A) RNA indicated major differences between virus types O and Asia 1. Base composition analysis of virus-induced RNA showed no significant differences between types O and Asia 1.

Keywords. Foot-and-mouth disease virus; poly(A) RNA; oligo(dT)-cellulose chromatography; oligonucleotide mapping; base composition analysis.

Introduction

Foot-and-mouth disease is an acute and highly contagious febrile disease affecting cloven-footed animals. Identification of the foot-and-mouth disease virus (FMDV), the causative agent of the disease, posed problems because of the occurrence of many types and subtypes of the virus. A molecular approach based on oligonucleotide mapping of FMDV RNA has been used for the identification and characterization of virus isolates obtained in a disease outbreak (King *et al.*, 1981). One-dimensional oligonucleotide mapping was used for rapid analysis of FMDV RNA (LaTorre *et al.*, 1982). FMDV types O and Asia 1 of Indian origin are being routinely used for vaccine production in India. This report presents the differences between FMDV types O and Asia 1 at molecular level based on one-dimensional oligonucleotide mapping of virus-induced poly(A) RNA.

Materials and methods

Actinomycin D, oligo(dT)-cellulose type T-2, ribonuclease T_1 and ribonuclease T_2 were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Carrier-free [^{32}P]-orthophosphate (activity 10 mCi/ml) was obtained from the Bhabha Atomic Research Centre, Bombay. All other chemicals used were of analytical grade.

Isolation of [^{32}P]-labelled poly(A) RNA from FMDV-infected BHK-21 Razi cells

BHK-21 Razi cells obtained from Razi Institute, Teheran, were grown to confluence

actinomycin D. At 2 h post-infection 2 mCi of carrier-free $[^{32}P]$-orthophosphate were added to each culture bottle. Medium was removed from cells infected with FMDV types O and Asia 1 at 6 and 8 h post-infection respectively and the cells were chilled in ice. RNA was isolated from the cells by the method reported earlier (Scodeller *et al.*, 1979; LaTorre *et al.*, 1982). Poly (A) RNA was isolated by oligo (dT)-cellulose chromatography as described by Grubman *et al.* (1979).

One-dimensional oligonucleotide mapping of FMDV-induced poly (A) RNA

Poly (A) RNA was digested with RNase T_1 by the method described by LaTorre *et al.* (1982). Digestion was carried out at an enzyme to substrate ratio of 1:20 for 60 min at 37°C. The oligonucleotides were separated on 8% polyacrylamide gel containing 8 M urea by one-dimensional electrophoresis using the method of Sanger and Coulson (1978) with minor modifications. The ethanol precipitate obtained from RNase T_1 digests was dissolved in 2 μl of dye marker solution containing 80% (v/v) formamide, 6 M urea, 0·2% bromophenol blue, 0·2% xylene cyanol and 1 M EDTA and the samples placed in the gel slots. The electrophoresis was carried out at 1200 V using TBE buffer, pH 8·3 (10·8 g of Tris, 5·5 g boric acid and 0·93 g of EDTA dissolved in distilled water, pH adjusted to 8·3, made up to 1 litre). After electrophoresis the gel was subjected to autoradiography at −70°C.

Base composition analysis

Base composition analysis of FMDV-induced RNA was carried out by the method described by Nishimura (1972). The $[^{32}P]$-labelled RNA was digested with RNase T_2 at 37°C for 20 h. Mononucleotides from the enzyme digest were separated by paper electrophoresis at 4000 V for 60 min using pyridine buffer, pH 3·5 (0·5% pyridine, 5% acetic acid and 5 mM EDTA). After electrophoresis the paper was exposed to X-ray film overnight at −70°C. The spot corresponding to each nucleotide was cut out and the radioactivity was determined in a liquid scintillation counter.

Results and discussion

Oligonucleotide maps of FMDV-induced poly (A) RNA are shown in figure 1. Six major bands, A, B, C, D, E and F present in FMDV type O were not detected in the case of Asia 1 virus. No difference in poly (C) tracks between FMDV types O and Asia 1 was observed. The results of base composition analysis of FMDV-induced RNA are shown in table 1. In both types O and Asia 1 RNA, cytidine monophosphate (CMP) was the predominant base and no unusual bases were present.

Oligonucleotide maps have indicated differences among FMDV subtypes A_{22}, A_5 and A_{24} (Robson *et al.*, 1979). Two-dimensional fingerprinting of RNase T_1 digests of viral RNAs from virus isolates made during an outbreak of foot-and-mouth disease in the UK, has helped to identify and characterize the isolates (King
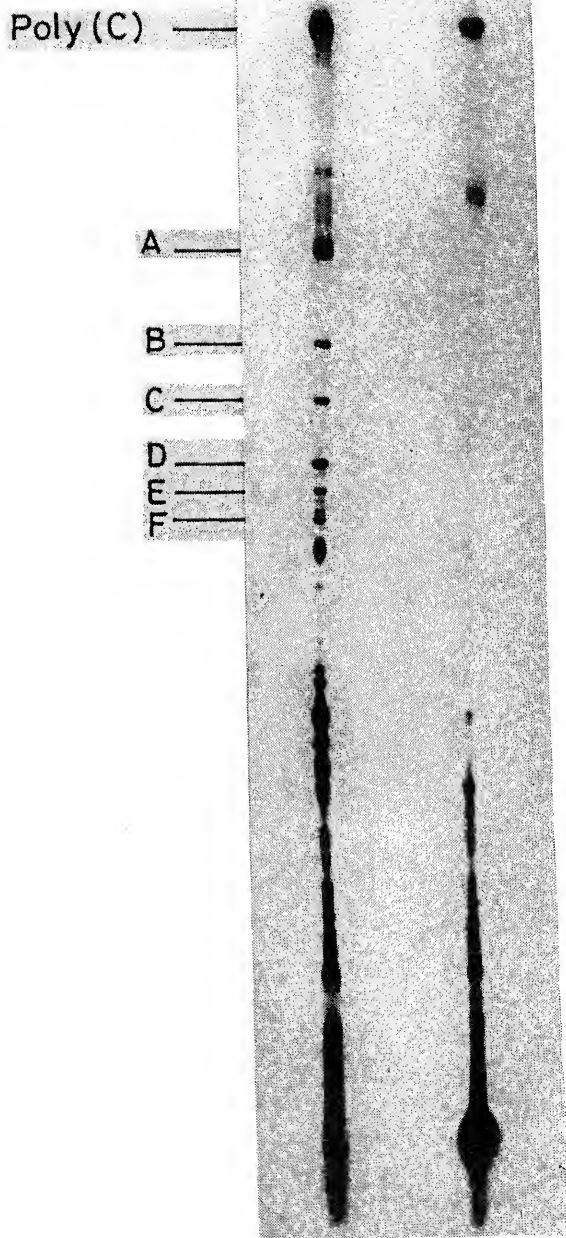
Figure 1. One-dimensional oligonucleotide mapping of FMDV types O and Asia 1 induced Poly (A) RNA. To each slot 50,000 cpm of oligonucleotides was applied. Lane I, FMDV type O; Lane II, FMDV Asia 1.

Table ... Base composition analysis of ... ... types ...
RNA.

Ribonucleoside monophosphate	Mole (%)	
	FMDV type O	FMDV type Asia 1
CMP	29·5	31·5
AMP	21·8	25·1
GMP	26·6	21·4
UMP	22·1	22·0
G+C	56·1	52·9

as a rapid method to distinguish between FMDV types and subtypes. This is often important in epidemiological studies particularly where the disease is endemic and antigenic variation is more likely. The present finding that CMP is the predominant base in FMDV types O and Asia 1 RNA is in agreement with that reported in the case of FMDV types A, O and C (Bachrach, 1977).

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Device for miniscale isoelectric focusing of proteins

H. S. PAWAR*, A. M. BODHE, M. V. RELE and H. G. VARTAK

Division of Biochemical Sciences, National Chemical Laboratory, Poona 411 008, India

Abstract. A simple device is developed for mini-scale electrofocusing of proteins. The main apparatus consists of only two glass tubes joined by a small tubing. No special cooling system, stopcocks, stands, etc., are needed. Even the need for a peristaltic pump for fractionation is eliminated. The apparatus does not require very high voltages and the amount of Ampholines is drastically reduced. The model can be used for analytical as well as semi-quantitative purposes.

Keywords. Isoelectric focusing; enzyme localization; Ampholine gradient; semiquantitative apparatus; analytical apparatus.

Introduction

Electrofocusing of proteins using ampholytes was first described by Svenson (1961a,b). Commercial units available for electrofocusing have disadvantages with regard to size, requirement of a cooling system and large amounts of Ampholines. Various small models have been described in literature (Weller *et al.*, 1968; Godson, 1970; Jackson and Russel, 1984). However, in most of these models, there is no provision for collecting the samples from the bottom which is not only most convenient but will also prevent disturbance of the pH gradient formed.

Materials and methods

Ampholines (pH range 3·5–10) were purchased from LKB Produkter, Sweden. Myoglobin and ferritin were from Sigma Chemical Co., St. Louis, Missouri, USA. Partially purified preparations of intracellular glucose (xylose) isomerase and extra-cellular xylose isomerase from *Chainia* sp. and subtilisin inhibitor from horse gram were from this laboratory. Haemoglobin from a *Bandicoot* sp. was a gift from Ahmednagar College, Ahmednagar. All other chemicals were of analytical grade and were available locally.

Description of the mini-electrofocusing apparatus

The apparatus consists of two pyrex glass tubes (7 mm × 35 cm). The lower ends of these tubes are fitted with two short (2 cm length) flexible silicone tubings. This is to facilitate the use of pinchcocks. These tubes are then connected to a U-tube made of a rigid material (such as polyethylene) of the same inner diameter (7 mm) as the glass tubes (figure 1). The platinum electrode in the heavy electrode solution is made longer (33 cm) in order to minimize resistance during the run. The other platinum
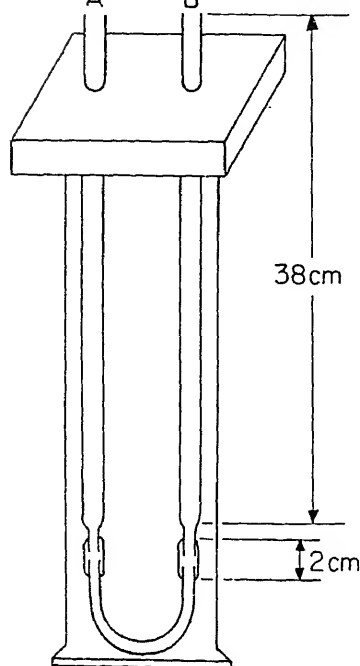
Figure 1. Schematic diagram of the modified electrofocusing set-up.

Solutions used for electrofocusing

Anode solution: Heavy electrode solution-glycerol 13·75 ml, H3P04 (1 M) 4 ml, H20 7·25 ml, total volume 25 ml.

Cathode solution: Light electrode solution-NaOH (1 M) 2·5 ml, H20 7·5 ml, total volume 10 ml.

Heavy density gradient solution: Ferritin 100 μg, myoglobin 250 μg, cytochrome c 100 μg, Ampholine (pH range 3·5–10) 40% solution 0·15 ml, glycerol 3 ml, total volume 5·4 ml with distilled water.

Light density gradient solution: Ferritin 100 μg, myoglobin 250 μg, cytochrome c 100 μg, Ampholine (pH range 3·5–10) 40% solution 0·15 ml, total volume 5·4 ml with distilled water.

Electrofocusing

The two arms of the tube assembly were inserted through a thermocole block and held vertically (figure 1). The heavy electrode solution was poured into arm A till it

light electrode (cathode) solution was then layered over the Ampholine gradient column. The pinchcock was removed and the assembled apparatus was immersed in a cylinder containing cold water at 4°C. Focusing was initiated by inserting the electrodes into the anodic and cathodic solutions and then applying a current of 4 mA at 400 V. Electrofocusing was complete in 27 h as indicated by the drop in amperage to zero. However, the run was continued up to 40 h to ensure the formation of a stable gradient.

Fractionation and collection of the Ampholine gradient

After the run a pinchcock was again fixed to the silicone tubing of arm B. The polyethylene U-tube was then disconnected from arm B. Fractions of 0·15 ml (approximately 3 drops) were collected by adjusting the flow rate suitably with the help of the pinchcock. The pH of each fraction was measured using a surface electrode and the individual fractions were assayed for protein or enzyme activity.

Results and discussion

Figure 3 shows a photograph of the apparatus after electrofocusing of ferritin, myoglobin and cytochrome c. The observed pI values of 10·3 for cytochrome c, 6·8-7·3 for myoglobin, 4·6- 5·1 for ferritin are close to the values reported in the literature. Intracellular glucose (xylose) isomerase, *Bandicoot* haemoglobin (3 bands), subtilisin inhibitor and extracellular xylose isomerase showed pI values of 4·0, 7·7, 7·85, 7·9; 7·66 and 3·5 respectively (figure 2).
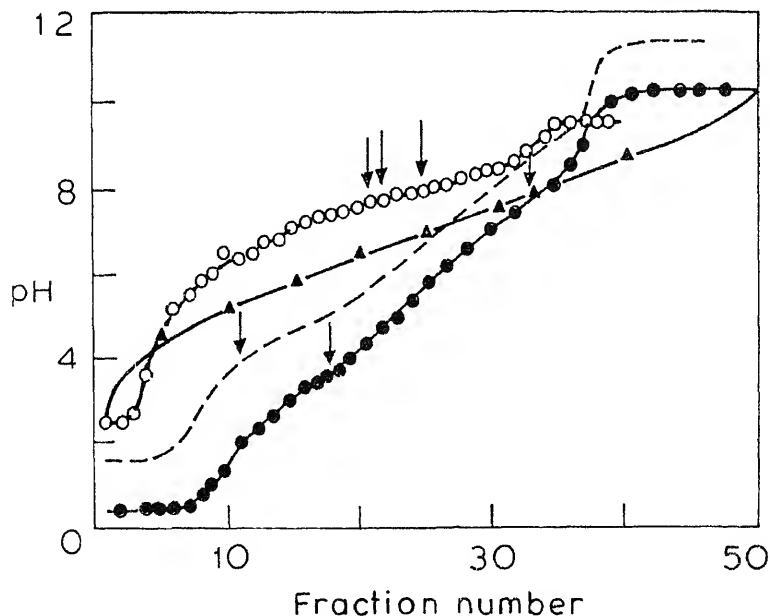
Figure 2. Determination of isoelectric point of ( ) intracellular glucose (xylose)
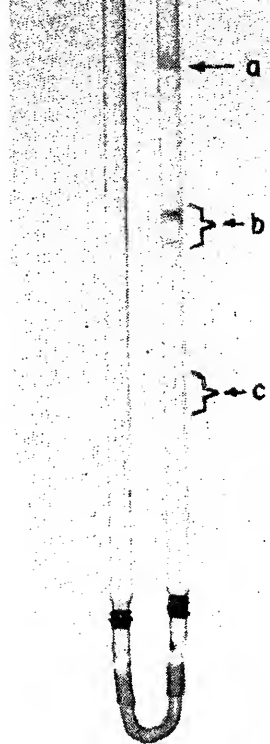
Figure 3. Isoelectric focusing pattern of (a) cytochrome c, (b) myoglobin (two bands) and (c) ferritin (two bands).

The U-tube apparatus described in this communication can be easily fabricated from locally available material. It is the simplest model to operate compared to those described in the literature. Since the entire apparatus is cooled and there is no capillary involved in the system (Jackson and Russel, 1984), very high voltages and consequent heating are eliminated in this model. The U-tube arrangement facilitates removal of fractions from the bottom and dispenses with the need for pumping in sucrose solution or the use of nitrogen to collect fractions from the top. The unit is being routinely used in our laboratory.

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Preparation of Concanavalin A-β-galactosidase conjugate and its application in lactose hydrolysis

SUNIL KUMAR KHARE and MUNISHWAR NATH GUPTA*

Chemistry Department, Indian Institute of Technology, New Delhi 110 016, India

Abstract. A Concanavalin A-β-galactosidase conjugate was prepared using glutaraldehyde as the crosslinking reagent. The conjugate bound to Sephadex G-50 beads was more thermostable and hydrolyzed lactose faster than the free enzyme. The immobilized enzyme may prove useful in the preparation of low lactose milk which is required by persons suffering from lactose intolerance.

Keywords. *Escherichia coli* β-galactosidase; ConA-β-galactosidase conjugate; lactose; lactose hydrolysis; low lactose milk.

Introduction

β-Galactosidase activity has been extensively studied in a large number of sources (Wellenfels and Weil, 1972). Undoubtedly, the main reason for this has been the use of this enzyme in the hydrolysis of whey (Kosaric and Asher, 1985) and hydrolysis of milk lactose for producing low lactose milk which is required by persons afflicted with lactose intolerance (Gekas and Lopez-Leiva, 1986). Both of these applications have encouraged the immobilization of β-galactosidase from various sources on a variety of matrices (Richmond *et al.*, 1981). In some cases, immobilized lactases have been used in commercial processes for hydrolysis of lactose in whey and milk (Pastore and Morisi, 1976). Nevertheless, the search for better enzyme derivatives continues (Makkar *et al.*, 1981; Friend and Shahani, 1982; Nakanishi *et al.*, 1983). Crosslinking an enzyme with a lectin to create a reusable enzyme derivative has been suggested as a possible alternative (Shier, 1985). In this paper, we describe the preparation of a conjugate of β-galactosidase with Concanavalin A (ConA) and consider its possible use in enzyme based bioreactors.

Materials and methods

Escherichia coli β-galactosidase was obtained from Sigma Chemical Co., St. Louis, Missouri, USA. ConA and *o*-nitrophenyl-β-D-galactopyranoside (ONGP) were procured from CSIR Centre for Biochemicals, Delhi. Commercial glutaraldehyde (25%) was a product of Riedel. All other reagents used were of analytical grade.

Assay of β-galactosidase activity

0.01 M Tris-acetate buffer, pH 7·5, containing 0·01 M MgCl$_2$); 0·75 ml β-mercapto-ethanol (1 M). The reaction was stopped after 5 min by adding 4 ml of 1 M Na$_2$CO$_3$ and the liberated o-nitrophenol was measured by reading the absorbance at 405 nm.

Enzyme activity of Sephadex-bound enzyme was also determined in a similar way: 200 μl sample i.e., 100 μl beads in 100 μl buffer (0·1 M sodium phosphate, pH 6·5, containing 0·003 M MgCl$_2$), was incubated with assay mixture with constant shaking.

Preparation of ConA-β-galactosidase conjugate

ConA-β-galactosidase conjugate was prepared using glutaraldehyde as bifunctional cross linking reagent. Solutions of ConA (4 mg/ml) and β-galactosidase (1 mg/ml) were prepared in sodium phosphate buffer (0·1 M, pH 6·5) containing 1 M NaCl and 0·003 M MgCl$_2$. ConA (500 μl) and β-galactosidase (100 μl) were mixed and were cooled to 4°C. A cold 25% aqueous glutaraldehyde solution (20 μl) was slowly added with constant mixing. The mixture was allowed to stand for 30 min at 4°C, after which it was directly loaded on a Sephadex G-50 column (1 × 15 cm) of 10 ml bed volume equilibrated with sodium phosphate buffer (0·1 M, pH 6·5) containing 0·003 M MgCl$_2$. The elution was carried out with 0·1 M NaCl in the same buffer. Flow rate was maintained at 22 ml/h and fractions of 1 ml were collected. Fractions containing protein and β-galactosidase activity were pooled and total protein and enzyme activity were determined.

The bound ConA-β-galactosidase activity was eluted using 0·2 M glucose in sodium phosphate buffer (0·1 M, pH 6·5) containing 0·003 M MgCl$_2$. Fractions of 1 ml were collected at a flow rate of 22 ml/h. The fractions containing conjugated β-galactosidase were pooled and total protein and enzyme activity were determined. Conjugation was also tried at pH 7; this resulted in precipitation of protein. The same result was obtained when β-galactosidase was increased to 200 μg in the reaction mixture and cross-linking was continued for 30 min. Hence in the latter case, attempts were made to obtain the conjugate by limiting the cross-linking time to 15 min (table 1).

Table 1. Optimization of conditions for preparation of ConA-β-galactosidase conjugate.

β-Galactosidase concentration (μg/reaction mixture)	Time of cross-linking (min)	β-Galactosidase activity in conjugate (%) of original
50	30	8·2
100	30	10·6
200	15	2·7

Protein was estimated by dye binding assay (Bradford, 1976) using bovine serum albumin as standard.

vities were determined at various concentrations of substrate. The K_m values were calculated after plotting the data according to Lineweaver and Burk (1934).

Lactose hydrolysis

One ml of lactose solution (5% in potassium phosphate buffer, 0·1 M, pH 7·2, containing 0·003 M Mg^{2+}) was incubated with 100 μl of the enzyme sample at 50°C. Aliquots of 100 μl were withdrawn at various times and their glucose content was measured by the PGO enzymatic method (Sigma Technical Bulletin, No. 510). These data gave the extent of lactose hydrolysis after various times of incubation.

Results

It has been suggested that enzyme–lectin conjugates may be useful derivatives for immobilization of enzymes (Shier, 1985). In the conjugate of β-galactosidase described here, ConA was chosen as the lectin component because this lectin is well characterized and easily available. The *E. coli* enzyme was chosen because its pH optimum around neutrality makes it an appropriate enzyme for milk lactose hydrolysis.

β-Galactosidase was cross-linked to ConA using glutaraldehyde. The conjugate was expected to bind to a Sephadex because of the affinity of the lectin to Sephadex column (Sharon and Lis, 1972). The binding and subsequent elution of the bound protein with 0·2 M glucose are shown in figure 1. The eluted protein consists of unreacted ConA and the conjugate of ConA with the enzyme. When enzyme concentration was varied, the best results were obtained at an enzyme concentration of 100 μg/620 μl of reaction volume (')le 1). The quantitative details of the recovery of enzyme activity at various stages are summarized in table 2. Thus 10% of the enzyme activity was recovered in the conjugate. The actual enzyme activity may in fact be
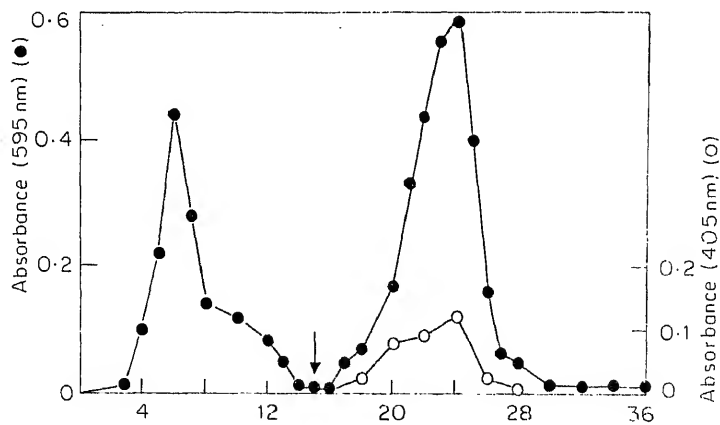
Table 2. Recovery of enzyme activity at various stages of preparation of the conjugate.

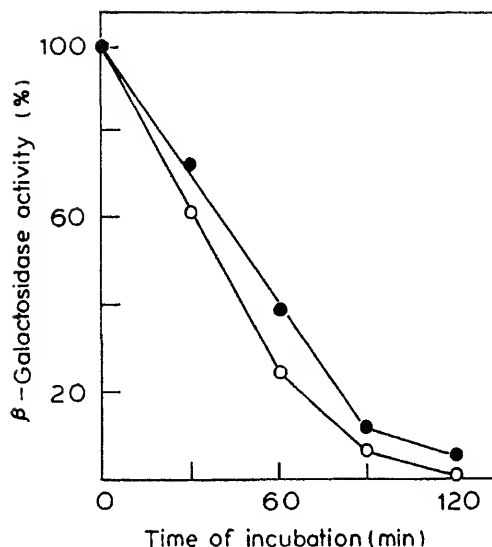| Sample	β-Galactosidase activity (%)
ConA-β-galactosidase solution	100
ConA-β-galactosidase solution, 30 min after adding glutaraldehyde	87
Effluent from Sephadex G-50 column eluted with 0·1 M NaCl	70
Effluent from Sephadex G-50 column eluted with 0·2 M glucose	10

slightly more since the measurement was made in the presence of glucose which
inhibitor of β-galactosidase (Deschawanne *et al.*, 1978). Dialysis to remov
glucose resulted in loss of enzyme activity. Such loss in enzyme activity on prol
dialysis in the case of β-galactosidase has also been reported by Rickenberg (
Since most of the activity was recovered in the initial washings with 0·1 M NaC
conjugation was not very efficient. There was also about 13% loss in enzyme a
upon conjugation. A similar loss in activity upon conjugation was observed
lactoseas substrate (Khare, S. K. and Gupta, M. N., unpublished results).

In this work, the unreacted ConA was not separated from the conjugate
enzyme is known to lose activity at low concentration and normally inert pr
are added in order to obtain a stable enzyme solution (Palmieri and Kold
1972). However, for further characterization, it should be easy to separa
conjugate from unreacted ConA with the help of a β-galactosidase affinity m
(Wellenfels and Weil, 1972).

The conjugate showed a marginal increase in thermal stability compared
native enzyme (figure 2).

However, the Sephadex-bound galactosidase conjugate showed consid
enhancement in thermal stability (figure 3). Many workers have studied the I
lysis of milk lactose by immobilized enzyme at 50°C (Friend and Shahani,
Nakanishi *et al.*, 1983). The conjugate bound to the Sephadex beads retained e
activity even after 12 h at 50 C (figure 3). At higher temperature (55°C) thou
bound conjugate showed enhanced thermal stability it gradually lost its activit
time (figure 3).

At low temperature (4°C), the bound conjugate again showed enhanced st
(figure 4). While there was no loss in enzyme activity after 12 days, only 10% e

Figure 2. Thermal stability profiles of free and ConA-conjugated β-galactosidase. Samples of free native β-galactosidase and ConA-β-galactosidase conjugate with identical activities were tested at 55°C; 2 mg ConA was added to the free enzyme solution. Aliquots of 200 μl were tested for activity after various times of incubation. (○), Free enzyme; (●), conjugate.
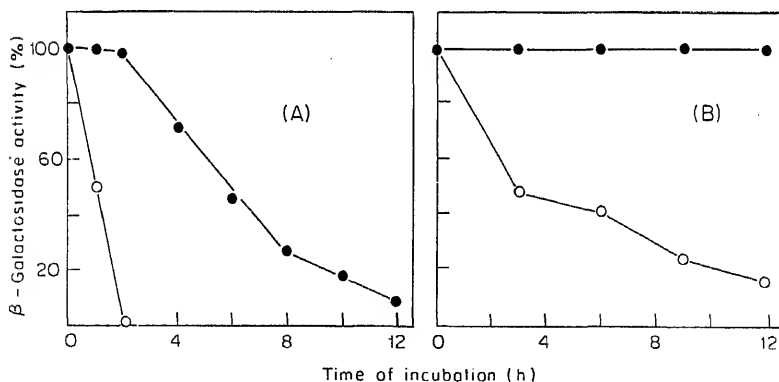
Figure 3. Thermal stability of Sephadex-bound ConA-β-galactosidase at 55°C (A) and 50°C (B). In each case, a free enzyme control with 2 mg ConA was also tested. (○), Control; (●), ConA-β-galactosidase conjugate.

The bound conjugate was used for lactose hydrolysis at 50°C (figure 7). It was found to be a more efficient biocatalyst compared to the free enzyme. Increased lactose hydrolysis by the bound conjugate is understandable since it was found to be a more thermally stable enzyme preparation compared to the free enzyme.
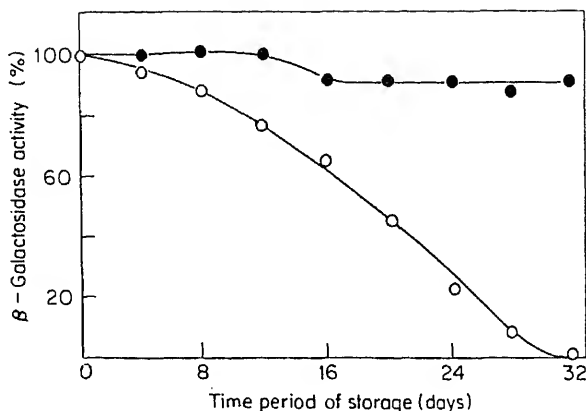
Figure 4. Effect of storage at 4°C on the β-galactosidase activity of Sephadex-bound ConA-β-galactosidase. (O), Control (as in figure 3); (●), Sephadex-bound ConA-β-galactosidase.
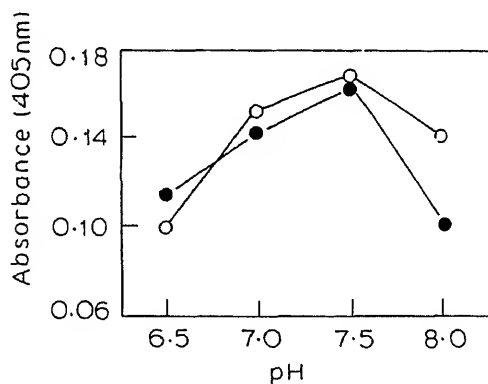
Figure 5. Effect of pH on enzymatic activity of Sephadex-bound ConA-β-galactosidase conjugate. The conjugate preparation and a free native enzyme control (as described in figure 3) were incubated at 25°C in sodium phosphate buffer (0·3 M) at different pH values. After 15 min of incubation, enzyme activity was determined using ONGP as substrate. (O), Free native enzyme; (●), Sephadex-bound ConA-β-galactosidase conjugate.

ConA). As the ConA-β-galactosidase conjugate is not very stable, it can not be used as a reusable enzyme derivative. However, the conjugate bound to Sephadex beads constitutes a useful reversibly immobilized lactase system. One possible disadvantage lies in the choice of ConA as a lectin (this choice was made because ConA is easily available in pure form), since when sufficient lactose is hydrolyzed the product glucose may reach sufficient concentration to dissociate the conjugate from the
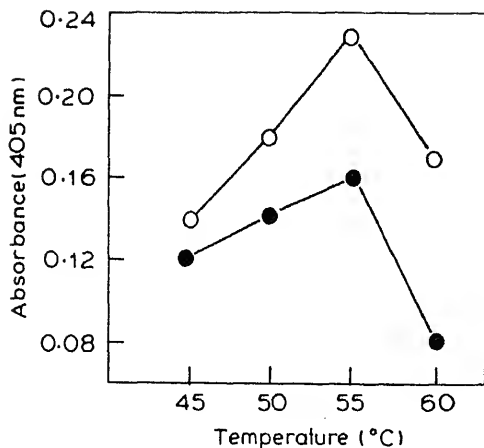
Figure 6. Effect of temperature on the enzymatic activity of Sephadex-bound ConA-β-galactosidase conjugate. The conjugate and free native enzyme (as described in figure 3) were incubated with the assay mixture (containing ONGP as substrate) at various temperatures. The enzyme activity was determined by estimating the liberated o-nitrophenol spectrophotometrically at 405 nm. (O), Free native β-galactosidase; ($\bullet$), Sephadex-bound ConA-β-galactosidase.

Table 3. K_m values of free and ConA-conjugated β-galactosidase.

	K_m (M)	
Sample	ONGP as substrate	Lactose as substrate
Free native enzyme	2.94×10^{-4}	5.43×10^{-3}
Sephadex-bound ConA-β-galactosidase conjugate	5.71×10^{-4}	9.77×10^{-3}

Reactions were as described in the test. Reaction with lactose as substrate was at 37°C.
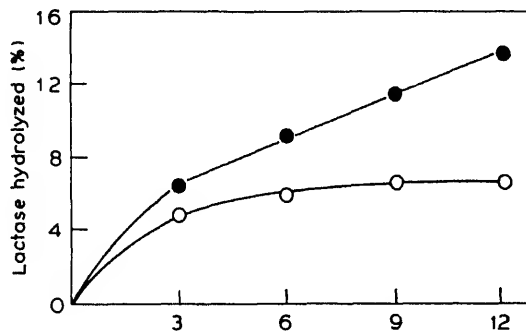
(i) Prepare the conjugate with another lectin in which case the recovery of the enzyme from reaction mixture would be made by using an affinity column.

(ii) Covalently link the *E. coli* enzyme to Sephadex directly or through a spacer or covalently link the ConA-β-galactosidase conjugate to Sephadex.

In this context, a recent paper by Solomon *et al.* (1986) may be mentioned where an approach somewhat similar to ours has been suggested as a novel method for immobilizing enzymes. Solomon *et al.* (1986) have used, instead of a lectin, an immobilized monoclonal antibody to bind the enzyme and immobilize it.

Finally, it may be mentioned that the ConA-β-galactosidase conjugate has one more potential application. It has already been reported that ConA-peroxidase conjugate can be used for staining of sciatic nerve glycoproteins on polyacrylamide gels (Wood and Sarinana, 1975). The product of the β-galactosidase reaction with ONGP is *o*-nitrophenol which is chromogenic. Thus ConA-β-galactosidase conjugate can be used like ConA-peroxidase to detect glycoproteins on polyacrylamide gels. Availability of more such conjugates may make this approach a more frequently used one for the detection and analysis of glycoproteins.

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Bioorganic chemistry of the purple membrane of *Halobacterium halobium*—Chromophore and apoprotein modified bacteriorhodopsins*

ANIL K. SINGH, M. ROY, S. SONAR and M. KAPIL

Department of Chemistry, Indian Institute of Technology, Powai, Bombay 400 076, India

Abstract. Iodophenyl and anthryl retinal analogues have been synthesized. The *trans*-isomers have been isolated and purified by high pressure liquid chromatography. The purified isomers have been further characterized by nuclear magnetic resonance and ultraviolet-visible spectroscopy. Incubation of these retinal analogues with apoprotein (bacterioopsin), isolated from the purple membrane of *Halobacterium halobium* gave new bacteriorhodopsin analogues. These analogues have been investigated for their absorption properties and stability. The iodophenyl analogue has been found to bind to bacterioopsin rapidly. The pigment obtained from this analogue showed a dramatically altered opsin shift of 1343 cm^{-1}. The anthryl analogue based bacteriorhodopsin, however, showed an opsin shift of 3849 cm^{-1}. It has been found that bacteriorhodopsin is quite unrestrictive in the ionone ring site. The apoprotein seems to prefer chromophores that have the ring portion co-planar with the polyene side chain.

The purple membrane has also been modified by treatment with fluorescamine, a surface active reagent specific for amino groups. Reaction under controlled stoichiometric conditions resulted in the formation of a modified pigment. The new pigment showed a band at 390 nm—indicative of fluorescamine reaction with amino group(s) of apoprotein —besides retaining its original absorption band at 560 nm. Analysis of the fluorescamine modified bacteriorhodopsin resulted in the identification of lysine 129 as the modified amino acid residue. Fluorescamine-modified-bacteriorhodopsin suspension did not release protons under photolytic conditions. However, proteoliposomes of fluorescamine-modified-bacteriorhodopsin were found to show proton uptake, though at a reduced rate.

Keywords. Bacteriorhodopsin; purple membrane; bacteriorhodopsin chemical modifications; proton pumping; bacteriorhodopsin analogues.

Introduction

Bacteriorhodopsin (bR) is a 26,000 dalton intrinsic membrane protein that functions as a light-driven proton pump in the purple membrane (PM) of *Halobacterium halobium* (Stoeckenius and Bogomolni, 1982). The discovery (Oesterhelt and Stoeckenius, 1971, 1973) of this pigment as the principal light-utilizing protein of Halobacteria growing under conditions of high salt and light intensity has provoked enormous interest, both in the protein itself and in the Halobacteria. bR has quite unique structural organization. It is arranged into extensive crystalline-like sheets consisting of tens of thousands of molecules tightly packed into hexagonal arrays. The smallest structural unit consists of 3 protein molecules, the trimers being separated from one another by a unimonolayer of tightly bound glycosulpholipid (Blaurock and Stoeckenius, 1971). Complete removal of the endogenous lipids from

bR and full recovery of proton translocating activity after reconstitution of the protein with added phospholipids have been accomplished (Huang *et al.*, 1980). The amino acid sequence of bR has been determined (Khorana *et al.*, 1979; Ovchinnikov *et al.*, 1979; Bayley *et al.*, 1981a; Wolber and Stoeckenius, 1984). The single chain of bR is composed of 248 residues, more than 70% of which are hydrophobic in nature. The lysine residue at position 216 is bound to a retinal chromophore *via* a protonated Schiff base (SBH$^+$) linkage (figure 1) (Lewis *et al.*, 1974; Bagley *et al.*, 1982; Rothschild and Marrero, 1982). Various attempts have been made to understand the general disposition of the protein within the membrane. Based on electron density maps it is believed that the polypeptide spans the membrane in 7 α-helical segments (Henderson and Unwin, 1975; Leifer and Henderson, 1983). In complete contrast, Jap *et al.* (1983), using circular dichroism and infrared spectroscopy, have suggested a model comprising 5 α-helices and 4 strands of β-sheet. The position of the chromophore has been refined by neutron diffraction studies using bR reconstituted with deuterated retinal (King *et al.*, 1979; Seiff *et al.*, 1985). Result of these studies place the β-ionone ring of the retinal near the centre of the membrane. It has been possible to assign the retinal site to one of two helical rods.
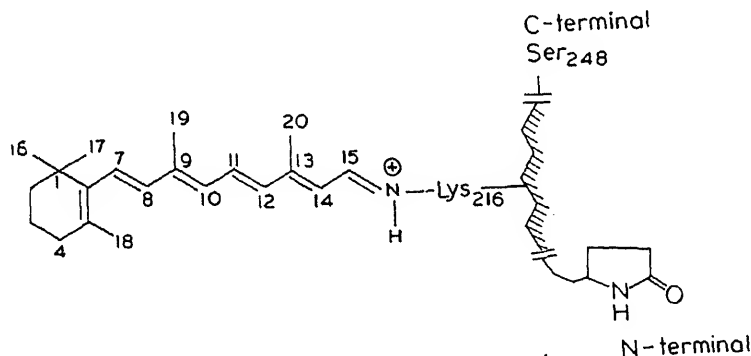
Figure 1.   all-*trans*-retinal bound to apoprotein *via* a protonated Schiff base linkage.

There are two forms of bR, the light-adapted (bR^{LA}) absorbing at 570 nm and the dark-adapted (bR^{DA}) absorbing at 560 nm, the chromophores of which are respectively all-*trans*-retinal and a 1:1 mixture of all-*trans*- and 13-*cis*-retinal. Both forms undergo a photocycle (figure 2). Absorption of light by bR drives the extrusion of H$^+$ ions from the cell to generate a proton gradient which can be utilized to fuel active transport and ATP production. Several mechanisms have been proposed, many involving the proton of the Schiff base, for the translocation process but they remain speculative. There has been considerable kinetic and spectrophotometric evidence to suggest that substantial conformational changes occur during the L to M transition (Kuschmitz and Hess, 1982) in which the Schiff base is deprotonated (Bagley *et al.*, 1982). Changes in the environment of tyrosine (deprotonation) and
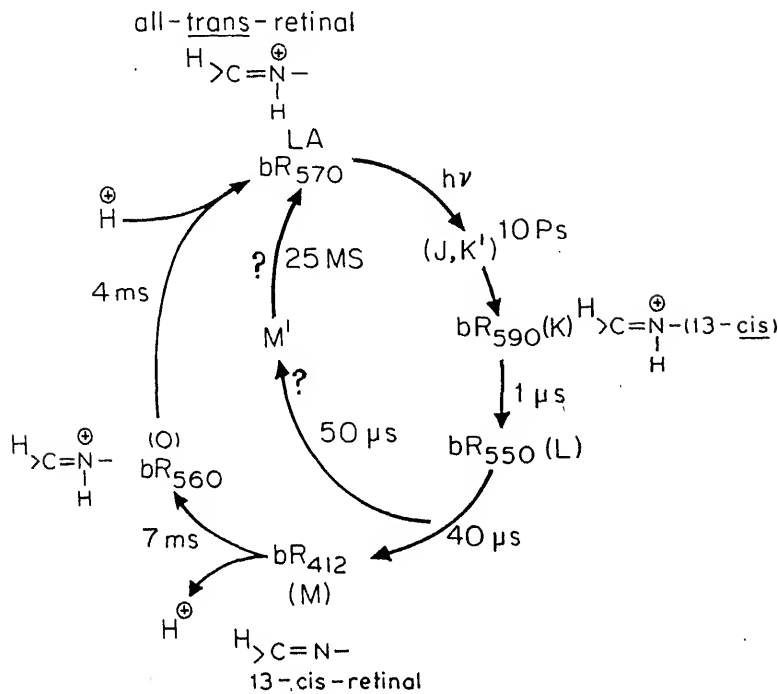
Figure 2. The photocycle of bR (Stoeckenius and Lozier, 1979; Stoeckenius and Bogomolni, 1982). Intermediate designations are shown in brackets; J, K' and M' have not been unambiguously established. The times shown refer to the particular transitions at room temperature. Subscripts denote the absorbance maxima (nm) of the various intermediates.

sidues 1–3, 68–72 and 231–248 are not vital for activity (Abdulaev *et al.*, 1978) rginines (Packer *et al.*, 1979) and carboxylic amino acids (Ovchinnikov *et al.*, appear to be important. Asp-115, in particular, which reacts with dicyclohexyl-diimide on exposure of the protein to light (Renthal *et al.*, 1985), has been sted to be critical to the proton pumping mechanism.

order to explain the marked báthochromic shift observed upon binding of al to the apoprotein, an experimental and theoretical analysis of the electronic onment of the binding site (Nakanishi *et al.*, 1980) positioned two negative ges (presumed to be carboxylate anions) in close proximity to the Schiff base and -ionone ring. Suggestions have recently been made (Lugtenburg *et al.*, 1986; ich *et al.*, 1986) that there may be a protein-bound positive charge in addition to ative charges in the vicinity of the β-ionone ring. This has opened an interest-ew chapter in our understanding of the chromophore-binding site, necessi-g further investigation.
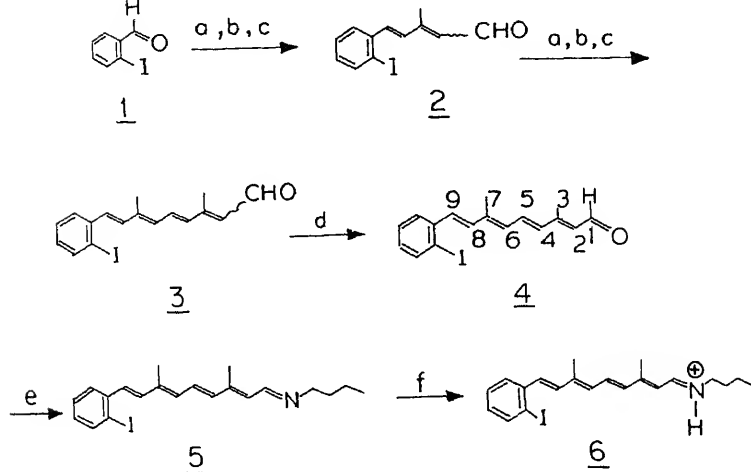
Scheme 1. Synthesis of iodophenyl analogue of retinal (*4*). a, C-5 Phosphonate, NaH, THF; b, LiAlH₄-EtO; c, MnO₂; d, HPLC; e, *n*-BuNH₂; f, dry HCl-MeOH.
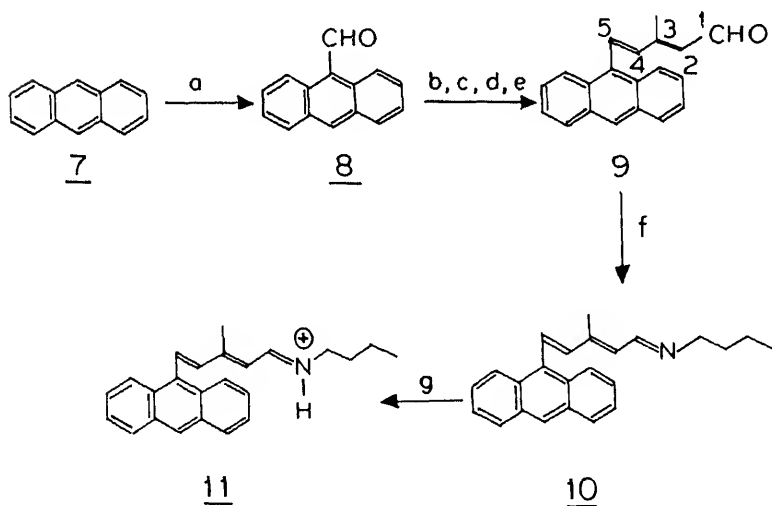
Scheme 2. Synthesis of anthryl analogue of retinal (*9*). a, DMF-POCl₃-*o*-dichlorobenzene; b, C-5 Phosphonate; c, LiAlH₄-Et₂O; d, MnO₂; e, HPLC; f, *n*-BuNH₂; g, dry HCl-MeOH.

electronic perturbations on the active site near the β-ionone ring. We have also modified bR with fluorescamine (FL) in order to probe the role of lysine residues in the overall structure and function of PM.

Materials and methods

Studies on retinals were carried out under dim red light and under N_2. The samples

r Aldrich, Fluka or Sigma make. Ultraviolet-visible (UV-vis) spectrophotometric
urements were made on a Beckman DU-6 spectrophotometer. Nuclear magnetic
ance (NMR) spectra were recorded on a Bruker WP-80 spectrometer using
l_3 as solvent and tetramethylsilane as internal standard. Ultracentrifugations
done on a Beckman L-8-55 M ultracentrifuge using SW-27 rotors. pH measure-
s were carried out on a Radiometer make pH meter (PHM-84) equipped with
2401C electrodes. High pressure liquid chromatography (HPLC) analyses were
rmed on a Beckman 110A HPLC instrument (microporacil, 10 μm Si-60,
250 mm, 1·5 ml/min, 9% ether-hexane, λ_{max} 360 nm, 254 nm). Lyophilizations
performed on Lyophilisers Pvt. Ltd., Bangalore instrument. Sonications were
on a Branson B-12 sonicator.

al analogue synthesis

l-4-(diethylphosphono)-3-methyl-2-butenoate (C_5 phosphonate) (Mayer and
1971) and activated manganese dioxide (MnO_2) (Fatiadi, 1976) were prepared
llowing the published procedures. Retinal analogues were synthesized using the
ons-type chain extension reactions to afford the esters, careful reduction with
H_4 to the alcohols, and oxidation to the desired aldehyde with activated MnO_2
mes 1 and 2). Pure all-*trans*-4 and 9 were obtained after HPLC analysis.
ted samples were stored at $-40°C$ under N_2 in dark for further use.

rotein preparation and its regeneration with synthetic retinals

er slants of *H. halobium* were kindly supplied by Dr. W. Stoeckenius, Depart-
of Physiology and Cardiovascular Research Institute, University of California,
Francisco, USA. Large-scale cultures were grown under illumination and low
en conditions. PM from the bacterial cells were isolated according to standard
ods (Oesterhelt and Stoeckenius, 1974). Bacterioopsin (bOP) was prepared by
hing the PM by irradiating with intense yellow light (Corning filter CS3-67 and
$CuSO_4$ solution in H_2O, 3 cm pathlength) in the presence of 0·75 M NH_2OH,
Bleached membrane protein was purified. The regeneration was achieved by
ddition of ethanolic solution of retinal analogues to purified bOP in HEPES
r, pH 7. The pigment proteins were purified and stored at $-40°C$.

tion of bR with FL

suspension in borate buffer, pH 9, and acetone solution of FL in 1:3
hiometry were stirred for 30 s at 4°C. The suspension immediately dialysed. The
nodified membranes were pelleted by centrifugation at 100,000 g. The
branes were lyophilized and then delipidated by dissolving in sodium dodecyl
ate and keeping overnight at 35°C. The protein (FL-bR) was precipitated by
ng ethanol. The pellet was washed several times with ethanol before diluting it
water. Ammonium hydroxide was slowly added and the resulting precipitate
stored at $-40°C$.

incubated at 37°C with chymotrypsin for 5 h. The membrane was collected
centrifugation (50000 g, 45 min) washed and lyophilized, and the fragments C-1
C-2 were separated on Sephadex LH-60.

Cyanogen bromide cleavage of chymotryptic fragment C-1

The fragment was dissolved in 88% (v/v) formic acid and treated with
cyanogen bromide (CNBr) (24 h, dark). The fragments were isolated by chrom
graphy on Sephadex LH-60 (88% formic acid-ethanol, 30:70).

Gel permeation chromatography

Gel permeation chromatography (GPC) was carried out on columns ($2·5 \times 80$ c
Sephadex LH-60 equilibrated in 88% formic acid:ethanol (30:70). Lyophi
membranes or fragments were dissolved in 88% formic acid, ethanol was added
ethanol concentration of 70% by volume, and the solution was used for chrom
graphy. The collected fractions were analyzed for their absorbance at 280
390 nm.

Proton translocation measurements

Proton release was measured on bR and fluorescamine-modified-bacteriorhode
(FL-bR) suspensions in 4 M aqueous KCl. Proton uptake was determined
proteoliposome preparations. The vesicles were prepared by the sonic meth
follows. PM and phosphatidylcholine (freshly isolated from hens' eggs) suspend
0·2 M KCl were sonicated in a Branson sonicator at 40 W power output in
bath for 3 min. The sonication was repeated 5 times with 2 min intervals.
resulting preparations were used for pH measurements under photolytic condi
($h\nu$, $\lambda_{max} > 500$ nm, 25°C).

Results and discussion

Retinal analogues

Synthetic analogues have been very useful tools for the elucidation of the stru
and mechanism of function of receptors. In the case of bR and rohodopsin, whic
photoreceptors, the interaction between retinal and opsin can be studied by in
gating the properties of pigment analogues formed from opsin and retinal analo
With this viewpoint we synthesized the all-*trans*-isomer of iodophenyl analogu
and anthryl analogue (9), which have different stereo-electronic features in the
portion of the chromophore. In addition, analogue 9 has a side-chain that con
only two ethylenic $C = C$ bonds, in contrast to the natural chromophore which

Table 1. Characteristic NMR signals for 4 and 9.

| Protons | Chemical shift (δ) (multiplicity and J values in Hz) | |
	4	9
H_1	10·16 (d, $J_{1,2}$, 10)	10·32 (d, $J_{1,2}$, 11·7)
H_2	6·0 (d)	6·18 (d)
H_4	7·60 (d, $J_{4,5}$, 16·5)	6·80 (d, $J_{4,5}$, 16·4)
H_5	7·15 (d, d, $J_{5,6}$, 12)	7·35 (d)
H_6	6·50 (d)	—
H_8	6·46 (d, $J_{8,9}$, 16·5)	—
H_9	7·80 (d)	—
3-CH_3	2·36 (s)	2·68 (s)
7-CH_3	2·16 (s)	—

[a]In $CDCl_3$, TMS, 80 MHz.

Table 2. Absorption maxima of chromophores and pigments.

| Compound | λ_{max}(nm) | | | |
	-CHO[a]	SBH$^+$[b]	pigment[c], LA	OS (cm^{-1})
all-*trans*-retinal	380	440	568 (560)[d]	5122
4	373	435	462 (455)[d]	1343
9	386	458	556 (550)[d]	3849

[a]Aldehyde; solvent, ethanol. [b]Solvent, methanol. [c]In 10 mM HEPES buffer, pH 7. [d]pigment, DA.
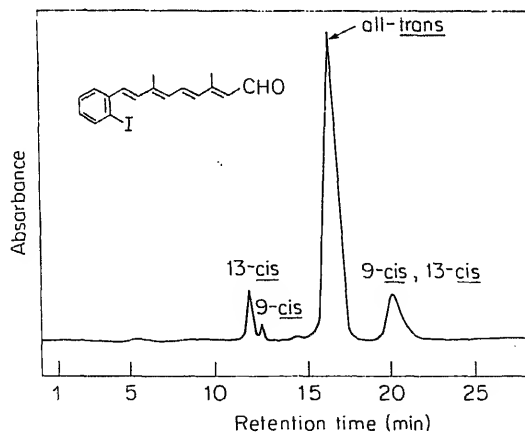
Figure 3. HPLC of synthetic mixture of iodophenyl analogue (4·5 mm × 25 cm micro-poracil, 1·5 ml/min, 9% Et_2O-HEX 360 nm).

Reconstitution of bR with retinal analogues
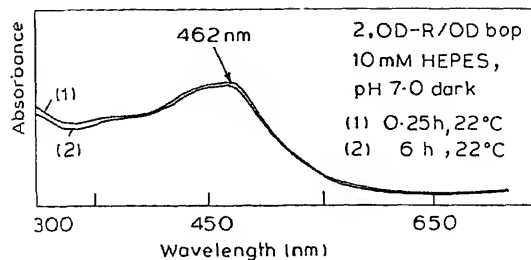
Figure 4.   Absorption spectra of bR analogue formed from iodophenyl chromophore ∴

minute of incubation. Similarly anthryl analogue 9 gave a new pigment w.··
absorption peak at 556 nm.

Opsin shifts

The opsin shift (OS) is a measure of the influence of the bOP binding site on t⁞
absorption spectrum of the chromophore. It is given by the difference between t⁞
λ_{max} of the SBH$^+$ of the chromophore in cm^{-1} and the λ_{max} of the pigment in cm
We prepared the SBH$^+$ 6 and 11 corresponding to analogues 4 and 9, respectiv⁞
with *n*-butylamine. For the sake of convenience the absorption spectra (figure 5)··
SBH$^+$ were measured in methanol (table 2). Next the model analogues were allow⁞
to bind to bOP, and the absorption spectra of the resulting pigments were obtaine⁞
The OS (table 2) for bR's with chromophores 4 and 9 were calculated to be 1343 a⁞·
3849 cm^{-1}, respectively.
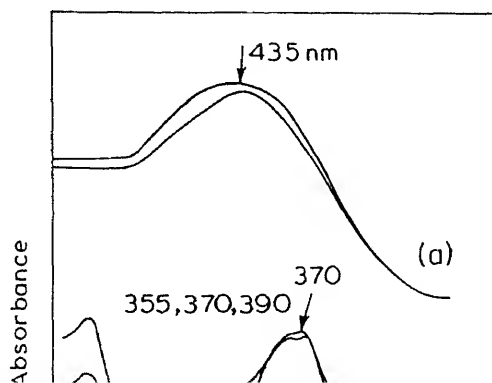
Dark-light adaptation

Samples of bR analogues in 2 M NaCl with 60% glycerol were cooled to 0°C and photolysed at $\lambda_{max} > 500$ nm for light adaptation. For dark adaptation, samples were put in dark overnight at 0°C. The bR-analogues showed dark-light adaptation with absorption maxima for dark-adapted (DA) bR-analogues 5–6 nm less than those for light-adapted (LA) bR analogues (table 2).

Identification of the pigment chromophore

The bound chromophore was extracted from the iodophenyl analogue based-bR with methylene chloride, vortexed for a minute and then centrifuging at 10000 g at 4°C. The methylene chloride fraction was analysed by HPLC. The analysis revealed the presence of all-*trans* isomer in the LA form and a mixture of 13-*cis* and all-*trans* isomers in the DA form. This indicated the presence of photocycle in the pigment.

Stability of pigment analogues

The reaction of the pigment analogues with hydroxylamine was measured by adding the reagent (1 mM, pH 7) to the pigment (<1 mg) and following the absorption spectrum over several hours in the dark. The pigments were found to be unstable in an excess of hydroxylamine.

An assessment of the binding site stability of the analogues was made by measuring of the displacement of the analogue chromophore upon addition of all-*trans*-retinal (0·2 mg/ml in ethanol) to the synthetic pigment. Thus the rates of binding of retinal and iodophenyl analogue of retinal to apomembrane were compared (figure 6). Such an analysis showed that the analogue *4* reacted much more rapidly, the formation of the chromophore being almost complete within the time (1 min) when the first measurement was made. Further, iodophenyl analogue bound to apomembrane was not readily displaced by all-*trans*-retinal. Thus, when retinal was added to bOP that had previously been treated with analogue *4*, the rate of reaction was reduced (figure 6).
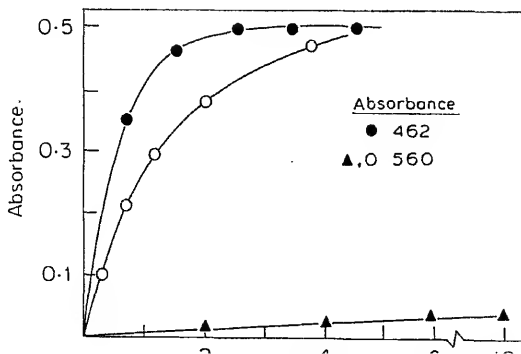
Previous studies (Mitsner *et al.*, 1986) have shown that numerous retinal analogues form pigments with bOP. The present investigations show that pigments can be formed with aldehyde side-chain containing even two ethylenic trans double bonds. Another finding of this work is that the iodophenyl analogue of retinal binds to the bOP more rapidly than retinal. This lends some support to the postulate that retinal binds in a sterically strained planar form (Schreckenbach *et al.*, 1977; Bayley *et al.*, 1981b) rather than in a conformation that is twisted about the 6–7 bond.

The reduced OS of iodophenyl analogue-bR (1343 cm^{-1}) in comparison to that of anthryl analogue-bR (3849 cm^{-1}) is because of a less planar conformation in the former. On the other hand the difference may simply be due to slightly different orientations of the two chromophores within the binding site and hence different influences from the external point charges (EPC) residing near the ring (figure 7).
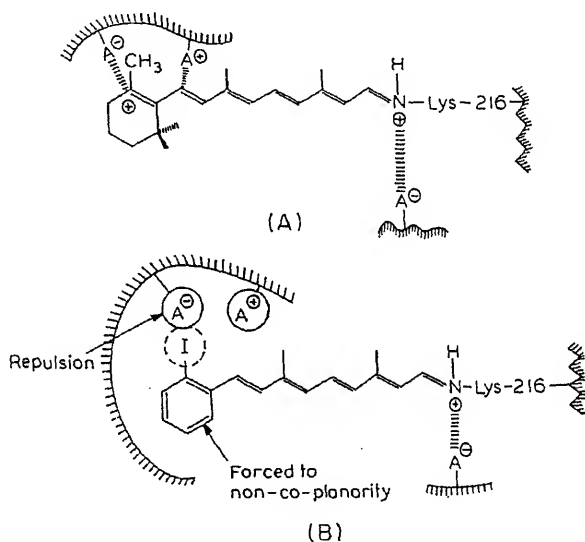
Figure 7. A. Model for bR binding site where 6-s-*trans* chromophore interacts with a pair of charges on bOP in the vicinity of β-ionone ring. B. Model for iodophenyl retinal analogue-bR, where due to soft, polarizable iodo group interaction with dipolar charge pair on bOP makes the phenyl group orientation non-co-planar.

According to the EPC model, electronic and/or steric perturbations at the ring binding site are expected to strongly affect the absorption maxima of bR-analogues. Therefore, the drastic deviations of the OS in the case of iodophenyl analogue of retinal lends support to the EPC model (Nakanishi *et al.*, 1980).

Though both chromophores *4* and *9* have an aromatic group with electronic features different from those of the natural chromophore. The phenyl chromophore exhibited rather small OS compared to the anthryl analogue, the anthryl chromophore may be considered as having some of the required double bonds in the tricyclic aromatic ring system; this imparts to the chromophore a more planar

Electronegative groups on the ring have been found to destabilize the excited state of the chromophore. Thus, the 5-trifluoro-methylretinal based bR-analogue has been found to show an OS of only 2400 cm^{-1} (Rao et al., 1986). The rather soft and polarizable iodo group on analogue *4* is expected to undergo electrostatic interactions with the opsin-bound pair of positive and negative charges in the vicinity of the ionone ring. The orientation of the ring will thus be governed by the resultant force of these interactions. The negative iodo group would like to keep away from the negative charge on the opsin. In addition, the migrating positive charge on the polyene in chromophore *4* would be repelled by the positive charge of the opsin (figure 7). The iodophenyl chromophore is influenced by these opsin-bound charges to adopt a non-co-planar conformation, leading to a reduced OS. Thus, in one way this model chromophore validates the very recent proposition that there is a protein-bound positive charge in addition to a negative charge in the vicinity of the β-ionone ring (Derguini et al., 1986; Lugtenburg et al., 1986; Spudich et al., 1986).

The OS of bR-analogue obtained from the planar anthryl chromophore *9* supports the recent hypothesis (van der Steen et al., 1986) that retinal binds to the apoprotein in its planar 6-s-*trans* conformation and not the 6-s-*cis* conformation.

Thus, in conclusion it can be said that the ring binding site in bOP is quite unrestrictive as even the highly modified anthryl and iodophenyl chromophores could be accomodated. Alterations to the side chain do not seem to prevent pigment formation. However, the apoprotein prefers a chromophore which has a ring site co-planar with the side polyene chain.

Modification of bR by FL

Chemical modifications of amino acid side chains can provide significant information regarding structural and functional features of the binding site. Various amino acid residues have been implicated in maintaining the structure and photo-biological functions of PM. bR pumps protons most effectively in the pH range 4 to 10. Possible groups involved in H$^+$ translocation by bR are the ε-amino groups of lysine, which have a pK of 10·5. Indeed schemes hypothesizing involvement of lysine in trans-membrane proton movement driven by pK shifts linked to Schiff base deprotonation have been postulated (Stoeckenius and Lozier, 1979). It has also been postulated that lysine groups play a structural role in maintaining a protein conformation that is essential for activity. Accordingly, it was of interest to evaluate the role of lysine residues by modifying them. We modified lysine residues of PM using FL, which is a surface-labelling and amino group-selective reagent. Treatment of reconstituted PM with FL has been shown to modify one lysine residue on the cytoplasmic side of the membrane (Tu et al., 1981).

Controlled treatment of PM suspension with FL in acetone at pH 9 resulted in a new pigment with absorption peaks at 560 and 390 nm (figure 8). Use of excess reagent and prolonged treatment gives a pigment with absorption at 500 and 390 nm; the 560 nm peak disappears, and denaturation of pigment occurs. The peak at 390 nm is characteristic for pigment formed by reaction of FL with −NH$_2$ groups of bR. The new pigment showed an emission peak at 472 nm. FL, as well as its
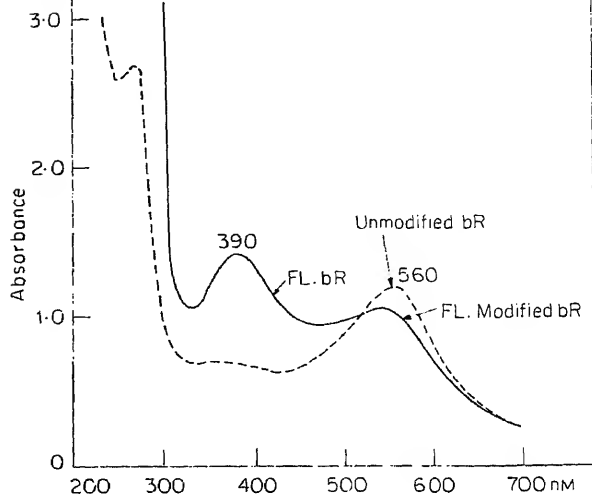
Figure 8. Absorption spectra of bR and FL-bR in suspension.

FL

$+$

—bR—(Lys-NH$_2$)$_6$
Bacteriorhodopsin

pH 9, 4°C
Suspension

—bR—(Lys)—N

OH

COOH

FLbR 12
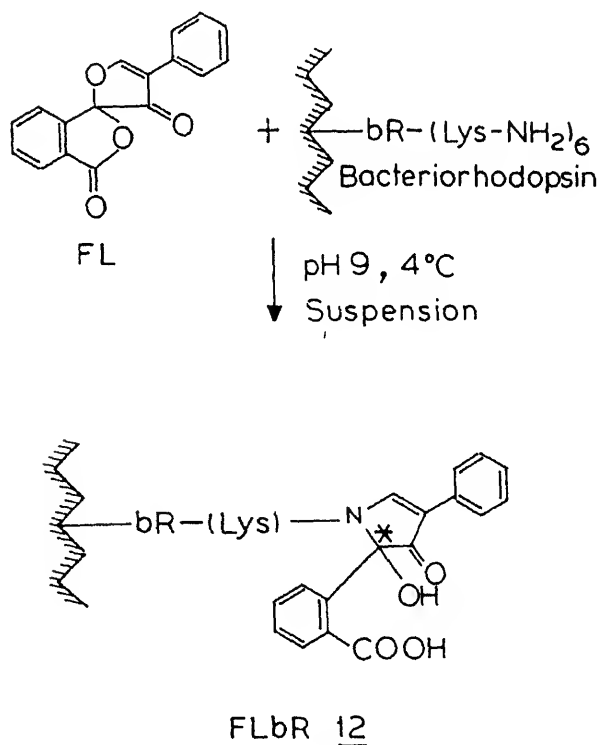
Scheme 3. Reaction of FL with bR.

Light-induced reaction of FL-modified bR

Light-induced H^+ uptake was measured using lipid vesicles with FL-bR or bR incorporated in the membrane. Proton uptake was determined to be 3 mol H^+ per mol of FL-bR and 6 mol H^+ per mol of bR. FL-bR, however, did not show light-induced H^+ release, thereby implying that bR cannot transport protons anymore when the light-induced proton release is blocked.

Identification of modified lysine

FL-bR was cleaved with chymotrypsin. Sephadex LH-60 GPC yielded fragments C-1 and C-2. Fragment C-1 was found to absorb at 390 nm indicating the presence of the FL modified lysine in this fragment. CNBr cleavage of C-1 followed by Sephadex separation of the fragments (figure 9) showed that fragment CNBr-7 contained the modified lysine residue (λ_{max} 390 nm).
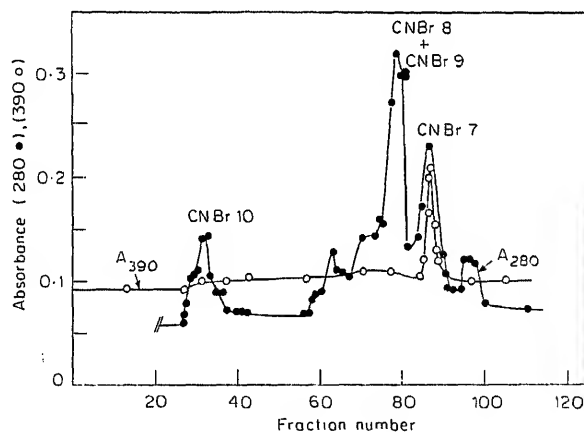
Figure 9. Chromatography of CNBr fragments of chymotryptic fragment C-1 of Fl-bR on Sephadex LH-60.

In principle CNBr cleavage of C-1 (residues 72–248) is expected to give 5 fragments (CnBr-6-10) (Gerber *et al.*, 1979; Huang *et al.*, 1982) (figure 10). As expected, CNBr-8 and 9 were co-eluted. CNBr-10 was eluted just after the void volume. CNBr-8 and 9 were followed by CNBr-7 in accordance with their sizes (figure 9). Unmodified bR fragments and FL-bR fragments were found to have similar elution pattern under the conditions employed. Thus, elution pattern does not change because of lysine modification by FL. CNBr-7 (residue 119–145) containing
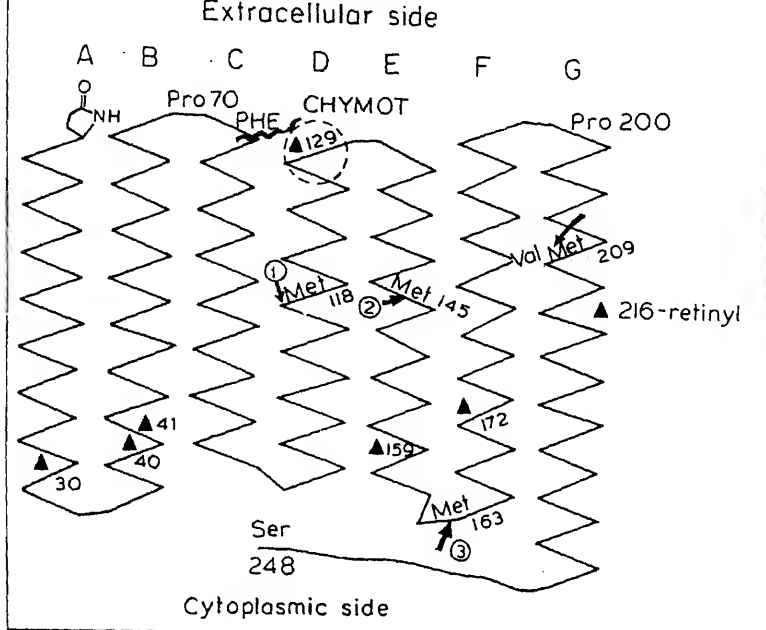
Figure 10. Primary sequence of bR showing lysine residues (▲) and sites of chymotrypsin (~ ~) and CNBr (→) cleavage sites in fragment C-1 (72–248)·Fl modified Lys-129 residue is circled. Retinal interacts with lysine-216.

proton pump mechanism. Lysine 216 is involved in retinal binding. The roles of lysine 30, 41 and 159 are yet to be established.

In conclusion, it can be said that chemical modification of bR or its apoprotein is capable of providing useful chemical information on the structural and functional properties of PM of *H. halobium*.

Acknowledgements

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R. V. HOSUR†, K. V. R. CHARY, A. SHETH, GIRJESH GOVIL and
H. T. MILES*

Chemical Physics Group, Tata Institute of Fundamental Research, Homi Bhabha Road,
Bombay 400 005, India

*National Institute of Health, Bethesda, Maryland 20892, USA

Abstract. New procedures have been described for accurate determination of solution structures of nucleic acids. These are two fold; new two dimensional nuclear magnetic resonance techniques and better approaches for interpretation of nuclear magnetic resonance data for structure determination purposes. The significant development in two dimensional nuclear magnetic resonance techniques for this purpose are ω_1-scaling and recording of pure phase spectra. Use of ω_1-scaled correlated and nuclear Overhauser effect spectra for estimation of interproton distances and ^1H-^1H coupling constants has been described. Computer simulation procedures for exact determination of structure have been described. Experimental spectra demonstrating the application of new procedures have been presented.

Keywords. Nucleic acids; ω_1-scaling; 2D NMR. computer simulation; inter proton distances.

Introduction

During the last 5 years, two-dimensional nuclear magnetic resonance (2D NMR) spectroscopy has been extensively used in an endevour to determine 3-dimensional structure of nucleic acids in aqueous solutions (Feigon *et al.*, 1983a,b; Haasnoot *et al.*, 1983; Hare *et al.*, 1983; Scheek *et al.*, 1983; Broido *et al.*, 1984; Clore and Gronenborn, 1985; Weiss *et al.*, 1984; Govil *et al.*, 1985; Hare *et al.*, 1985; Ravikumar *et al.*, 1985; Chazin *et al.*, 1986; Frechet *et al.*, 1983; Hosur, 1986; Hosur *et al.*, 1985a, 1986a,b; Chary *et al.*, 1987; Sheth *et al.*, 1987a, b). A fair amount of success has been achieved and it has been possible to assess qualitatively the sequence-specific structural features in oligonucleotides of lengths of 10–15 units. In short the procedure for structure determination involves (i) the use of cross peak positions or coordinates in 2D J-correlated (COSY) spectra (Jeener, 1971; Aue *et al.*, 1976) and nuclear Overhauser effect (NOE) correlated (NOESY) spectra (Jeener *et al.*, 1979; Anil Kumar *et al.*, 1980; Macura and Ernst, 1980; Macura *et al.*, 1982) to obtain sequence-specific resonance assignments of protons in DNA fragments and (ii) interpretation of relative intensities of cross peaks in the two types of spectra to derive structural information about the molecule. The NMR parameters relevant for this purpose are the coupling constants and NOE intensities and these are related to dihedral angles and interproton distances, respectively.

The J-correlated spectra provide information about J-coupling networks and enable identification of chemical shifts of sugar ring protons. In addition the H6 and CH$_3$ protons of thymines and H6 and H5 protons of cytosines in the oligonucleotide can be readily identified. The NOE correlated spectra on the other hand, reflect through-space interactions between protons and thus show distance correlations between protons on the same nucleotide as well as between protons on adjacent nucleotide units. These correlations are useful for obtaining sequence-specific assignments.

The intensities of cross peaks in NOE correlated spectra primarily depend on the interproton distances. They also depend to a substantial extent on the experimental parameters. Of particular importance is the mixing time (τ_m) used in the pulse sequence of NOESY. During this time, magnetization transfer occurs between protons coupled through dipolar interactions (D). If the system consists of only two cross relaxing protons, then the intensity of the cross-peak increases with mixing time till a steady state is reached. However, when the system consists of more than two protons, it constitutes a network of D-coupled spins and a more complicated diffusion of magnetization occurs. This is referred to as spin diffusion. Under such conditions, the relative intensities of NOESY cross peaks are not a true measure of the distances between the protons they connect. Therefore, experiments should be performed with low mixing time for reliable use of intensities for distance estimation. Under these conditions, appearance of a cross peak implies an interproton distance of less than 4 Å.

Another important factor which affects intensities is the line shape. The line shapes are distorted when complex data manipulation procedures are employed, and in the event of poor multiplet resolution, component intensities cancel each other, resulting in poor overall intensities. Procedures have been evolved to overcome these problems and some success has been achieved in getting reasonable estimates of interproton distances (Hare *et al.*, 1985, 1986a, b; Chary, K. V. R., Hosur, R. V. Govil, G. and Miles, H. T., unpublished results). Methodologies are being still developed for obtaining more accurate distance estimates. Overlap of cross peaks has often restricted the number of measurable distances in the molecule.

In the case of J-correlated COSY spectra, the cross peaks which contain the coupling constant information have anti-phase character. Under conditions of low resolution in the spectrum (caused by limited disk storage space, spectrometer time, etc), the component intensities cancel resulting in poor overall intensities. Since the cancellations depend upon the magnitudes of the coupling constants involved, the relative intensities in a given COSY spectrum can be used to derive qualitative information about the magnitudes of the coupling constants. The vicinal coupling constants are related to the dihedral angles in the sugar ring and thus help in fixing the geometry of the sugar ring.

A more precise definition of the structure of a DNA segment can be obtained by extracting detailed NMR information and following the procedures outlined below.

(i) Exact knowledge of coupling constants to fix the sugar geometries.

(ii) Accurate knowledge of a large number of interproton distances from NOESY

(iv) Energy minimisation to remove short contacts and obtain the preferred conformation.

While considering any of the 4 steps mentioned above, one has to keep in mind the dynamics of the molecule. The observed parameters are time averages and therefore the data will have to be analyzed in terms of contributions from individual conformers. However, it is observed that in large oligonucleotides (10–15 units long), the central units do not exhibit too much motion in the sense that only one conformer makes a major contribution to the observed properties. In the following, we describe new developments in our laboratories which will take us a long way in the determination of 3D structures of nucleic acids in aqueous solutions. We first describe the new 2D NMR techniques termed "ω_1-scaling techniques" which have helped in achieving sensitivity and resolution enhancement in 2D NMR spectra. This is followed by methodologies for interproton distance estimation and measurement of coupling constants. Finally, we discuss computer simulation of NOESY spectra for overall comparision of spectra. Clearly these steps must follow the resonance assignment, procedures for which are fairly well established.

New techniques in 2D NMR

Among the recent developments that have taken place in 2D NMR, two important ones have proved very useful in the study of biological molecules in solution.

(i) Recording of phase sensitive spectra instead of absolute value plots has greatly improved resolution (States *et al.*, 1982; Marion and Wuthrich, 1983). In this process the dispersive components which have very long tail and therefore hamper resolution are eliminated.

(ii) ω_1-*scaling in 2D NMR spectroscopy*: This concept has originated very recently (Brown, 1984; Hosur *et al.*, 1985b) but it has already found wide application (Gundhi *et al.*, 1985; Ravikumar *et al.*, 1986; Sheth *et al.*, 1986; Hosur *et al.*, 1987a). It can be used for sensitivity enhancement, resolution enhancement, ω_1-decoupling, narrowing of diagonals, measurement of coupling constants, long range correlation, etc.

A combination of these two developments has several advantages and will be the technique of choice in the future. For example, figure 1a shows the basic ω_1-scaling pulse scheme which can be incorporated in all forms of correlated spectroscopy such as spin echo correlated spectroscopy (SECSY) (Nagayama, 1980), relayed COSY (Eich *et al.*, 1982), NOESY, Z-COSY (Oschkinat *et al.*, 1986), etc. and also in 2D multiple quantum spectroscopy (Braunschweiler *et al.*, 1983). In every case, the evolution period including the two end radiofrequency pulses is replaced by the pulse scheme of figure 1a. The experimental procedures such as phase cycling have to be suitably modified. The pulse scheme of figure 1a achieves chemical shift scaling by a factor α and J-scaling by a factor γ along ω_1-axis of the 2D spectrum. It is obvious from the scheme that γ must necessarily be larger than α. Within this constraint, both the factors can be selected in a manner most appropriate for the experiment being performed. For example, selecting γ to be less than unity in NOESY results in both resolution and sensitivity enhancements, whereas in COSY it can lead to loss of cross
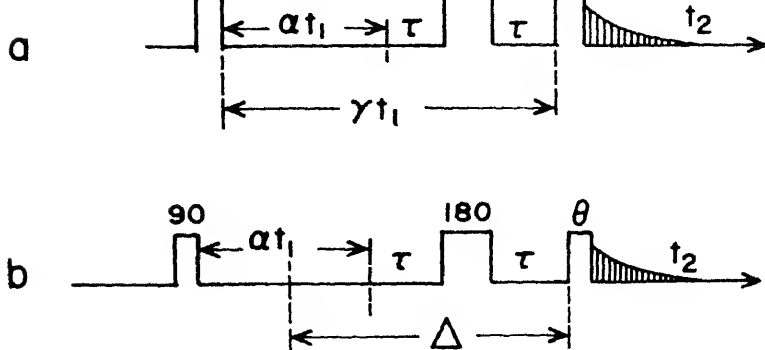
Figure 1. Pulse schemes for phase sensitive ω_1-scaled COSY (a) and exclusive shift scaling (b). The latter is termed as COSS (Hosur *et al.*, 1985c). α and γ are chemical shift and J-scaling factors respectively, τ is a delay which changes with t_1, Δ is a fixed delay and θ is the flip angle of the mixing pulse.

resolution and sensitivity enhancements. In general, $\alpha < 1$ helps in increasing multiplet resolution within the peaks in the 2D spectrum. However, it reduces the separation between the peaks and therefore α has to be optimally chosen so as not to merge the peaks.

Figure 2 shows the improvement in multiplet resolution obtained by the ω_1-scaled phase sensitive COSY (figure 2b) over the conventional absolute value COSY (figure 2a) spectrum for the oligonucleotide d-CTCGAGCTCGAG. From figure 2b it is possible to measure coupling constants as indicated in the figure. Both spectra have been recorded in about the same experimental time (about 15 h). Figure 3 shows the result of incorporation of the scheme of figure 1 in the NOESY pulse scheme. This ω_1-scaled phase sensitive NOESY spectrum has significantly higher resolution and sensitivity compared to conventional absolute value NOESY spectra. This allows recording of good quality spectra with a large number of cross peaks even at very low mixing times. This is a crucial factor for structure elucidation purposes. In the spectrum of figure 3, mixing time is 200 ms and a large number of intra and internucleotide cross peaks are seen. The cross peak regions have been identified by the proton labels in the figure.

Figure 1b shows a pulse scheme (correlation with shift scaling, COSS) (Hosur *et al.*, 1985c) for exclusive scaling up of shifts in the 2D correlated spectrum, which is not possible with the pulse scheme of figure 1a. Here again the shift scaling factor can be chosen arbitrarily. The purpose of this experiment is to increase the separation between cross peaks in the 2D spectrum. Since the J-values are not scaled, the sizes of the cross peaks are not altered significantly except for small changes due to slight increases in linewidths as a result of scaling. This effectively results in an increase in the separation between the peaks. As far as dispersion of the peaks is concerned the experiment is equivalent to performing a COSY experiment on a higher field spectrometer.

The parameter Δ in figure 1b alters the phase characteristics of the cross and

d- C T C G A G C T C G A G
   1 2 3 4 5 6 7 8 9 10 11 12
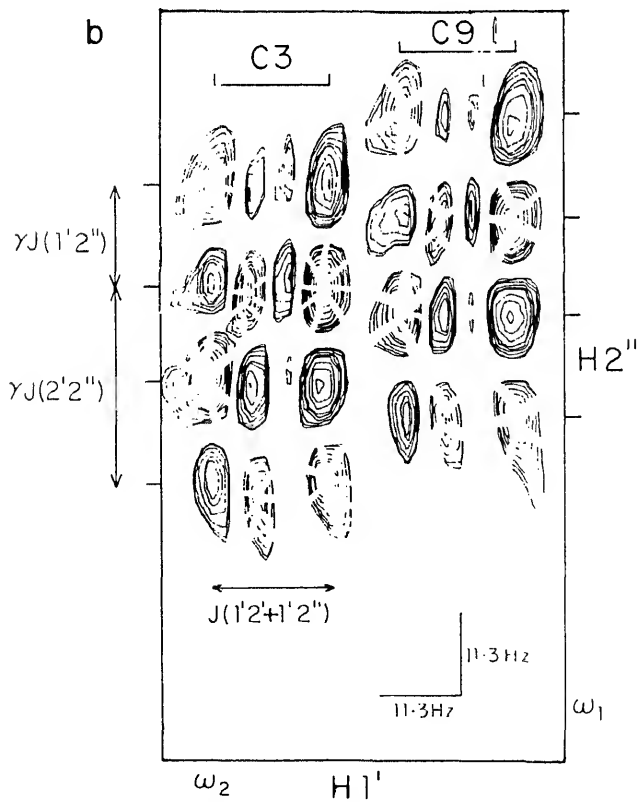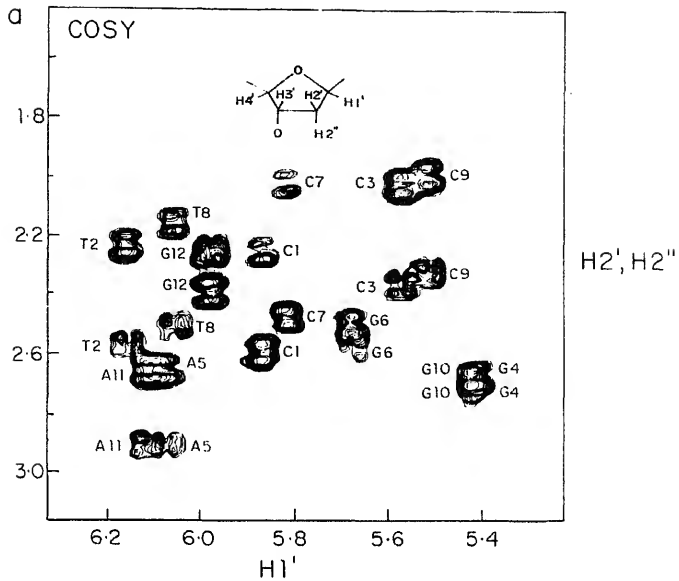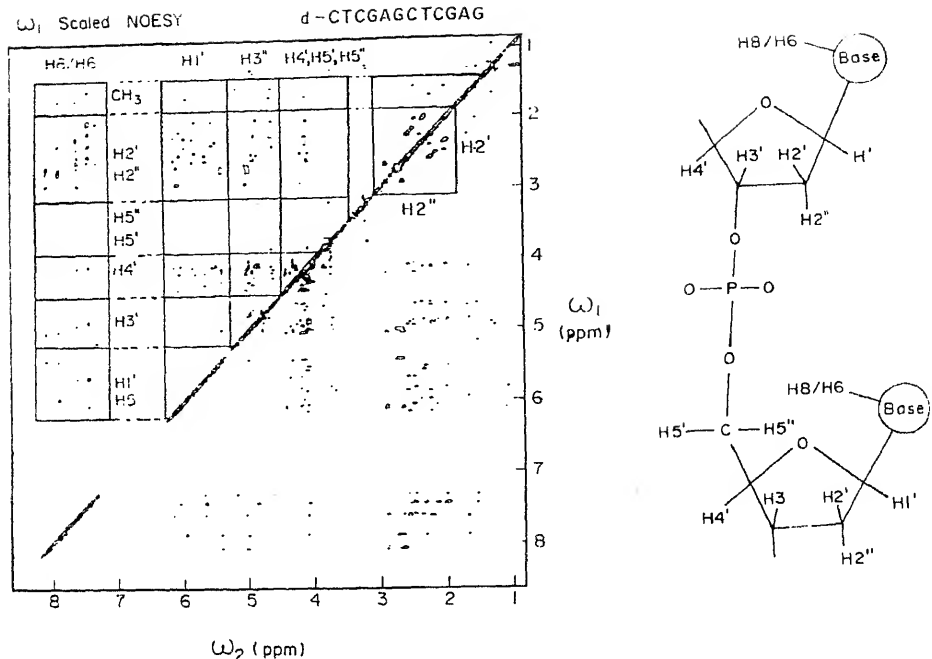
Figure 3. ω_1-scaled NOESY spectrum of the same sample as in figure 2. The cross peak regions have been identified by the hydrogen labels given near the individual boxes. A dinucleotide fragment showing nomenclature of atoms is also included. Many of the boxes contain cross peaks between protons on the same nucleotide as well as between adjacent nucleotide units.

obvious. However, the COSS technique has one disadvantage which arises because of the alteration of phase characteristics of the peaks. Both diagonal and cross peaks acquire contributions from dispersive as well as absorptive components. The dispersive components tend to reduce the resolution within the peaks, and this is undesirable for obtaining J coupling information. Therefore the application of COSS will be in resolving ambiguities due to overlap of cross peaks rather than in improving multiplet resolution which is the case with ω_1-scaled phase sensitive COSY.

Estimation of interproton distances

As mentioned in the introduction, the intensity of a NOESY cross peak at low mixing time (below the spin diffusion limit) depends directly on the interproton distance. Quantitatively, the intensity is proportional to cross relaxation rate σ_{ij} between the two protons i and j and this is inversely proportional to the sixth power of distance r_{ij} as shown below
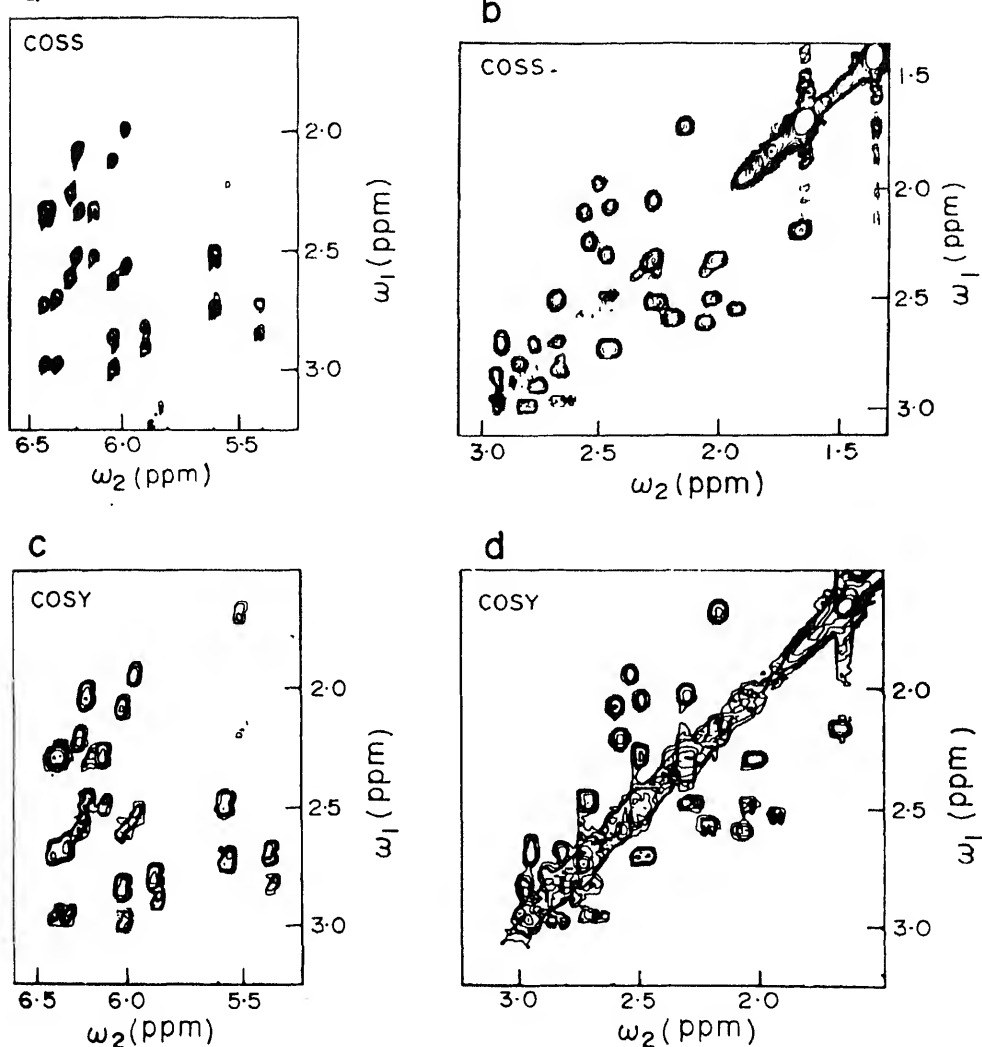
Figure 4. Comparison of 500 MHz absolute value COSY (c, d) and COSS (a, b) spectra in two different regions. Improved resolution and narrow diagonal can be seen in COSS spectra (a and b). Value of α is in 1·5 in 'a' and 2 in 'b'.

Under the conditions of slow motion $(\omega\tau_c \gg 1)$ prevalent in biological macromolecules, a simplification arises and the intensity is given by (Ernst *et al.*, 1987)

$$I_{ij} \propto \frac{\tau_c \tau_m}{r_{ij}^6}. \tag{2}$$

Assuming that the correlation time τ_c is the same for all proton pairs, one can obtain

which in principle can be different for different protons. NOESY experiments must be performed with various mixing times and NOE build-up curves obtained as functions of mixing time. In the 2D spectra, NOE intensities correspond to volumes of the peaks and different kinds of approaches have been adopted to estimate the volumes of the peaks in the 2D NOESY spectrum. The simplest of these is indicated in figure 5a. It is assumed that the peaks have absorptive Lorenzian line shapes along both the axes, an assumption which is valid under the conditions in which phase sensitive spectra are recorded employing weak data apodization functions. Volume is then calculated as

$$V \alpha \pi \times L_1 \times L_2 h, \tag{4}$$

where h is the height of the peak and $2L_1$ and $2L_2$ are the widths at half-heights along the ω_1 and ω_2 axes respectively (figure 5a). Figure 5b shows typical cross sections along the ω_1 and ω_2 axes through a few cross peaks in the phase sensitive NOESY spectrum of d-GGTACGCGTACC.
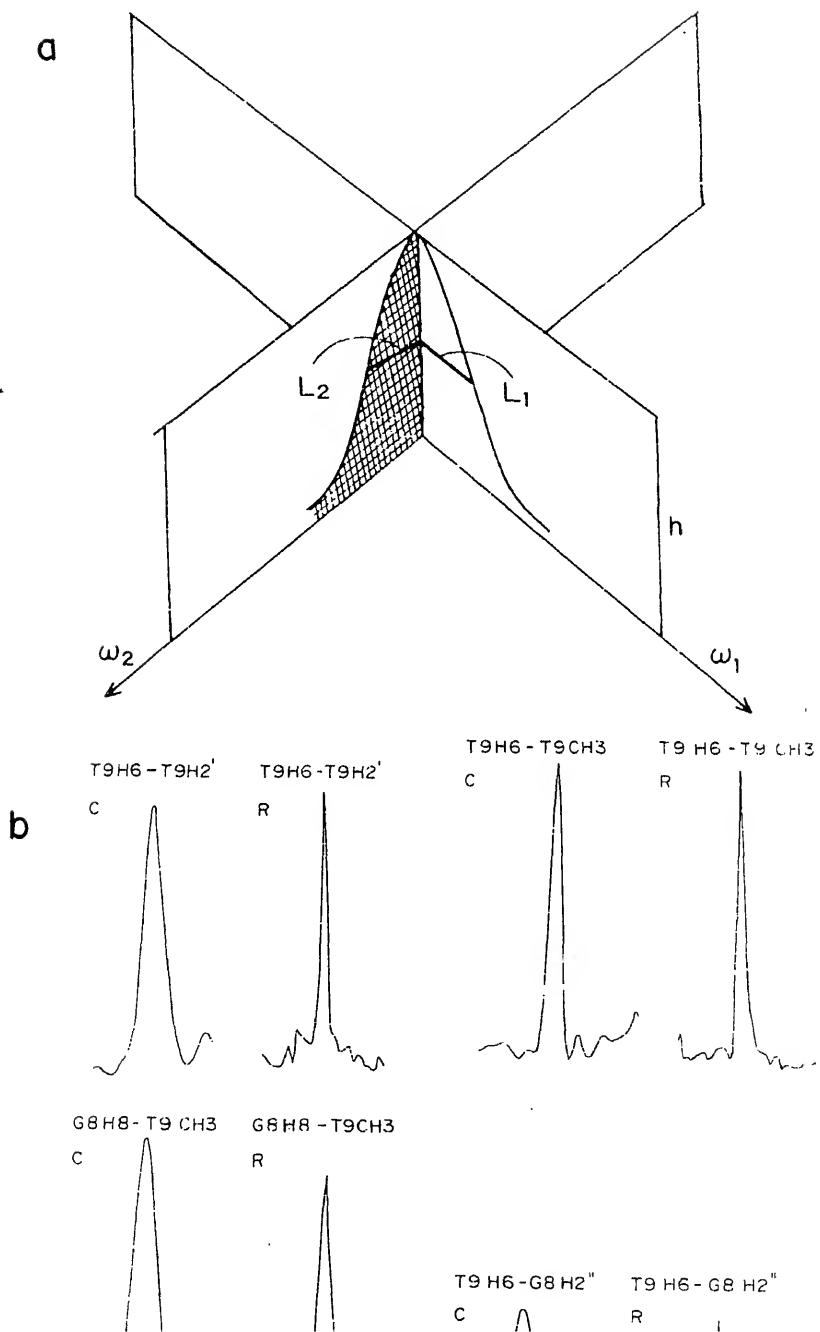
In the absence of spin diffusion there will be linear dependence of NOE on τ_m. As spin diffusion sets in, different types of NOE build-up curves are obtained. Figure 6 shows some illustrative curves obtained in the case of d-GGTACGCGTACC and it is seen that the patterns are different for different proton pairs. From these curves it is clear that one has to use a τ_m of less than 80 ms to draw meaningful conclusions about distances. Following the procedure described above several interproton distances have been measured and some of these are shown in figure 7. Details will be published elsewhere.

Measurement of ^{1}H-^{1}H-coupling constants

The protons of d-ribose rings constitute a complex network of coupled spins and each one of them has a complex multiplet structure. There are 6 observable coupling constants, namely $J(1'2')$, $J(1'2'')$, $J(2'3')$, $J(2''3')$, $J(3'4')$ and $J(2'2'')$. Their values depend critically on the geometry of the sugar ring, and the relationship has been analyzed earlier (Hosur, 1986; Hosur et al., 1986a, b; Rinkel and Altona, 1987).

The best method to obtain coupling constants is from the analysis of cross peak multiplet patterns in the ω_1-scaled phase-sensitive COSY spectrum (Hosur et al., 1987b). The expected patterns for a few illustrative cases are shown in figure 8; these are valid for $\theta = \pi/2$ in figure 1a. If $\theta < \pi/2$, then the cross peaks look simpler with fewer components, but this simplification is at the cost of some sensitivity in the spectrum. Thus the ω_1-scaled COSY should be optimised with judicious choice of θ, α and γ depending upon the needs.

Figure 9 shows H1'-(H2', H2'') spectral regions of ω_1-scaled phase sensitive COSY spectrum of d-CTCGAGCTCGAG, with $\theta = \pi/4$. The cross peak components are seen to be well resolved enabling measurements of $J(1'2'')$, $J(1'2')$ and $J(2'2'')$. It is apparent that $J(2''3')$ is less than the width of each peak along the ω_1-axis. $J(2'3')$ can be obtained indirectly from H1'-H2' cross peaks (upfield peak for every H1' except

a

L_2 L_1

h

ω_2 ω_1

b

T9H6 – T9H2'
C

T9H6 – T9H2'
R

T9H6 – T9CH3
C

T9 H6 – T9 CH3
R

G8H8 – T9 CH3
C

G8H8 – T9CH3
R

T9 H6 – G8 H2"
C

T9 H6 – G8 H2"
R
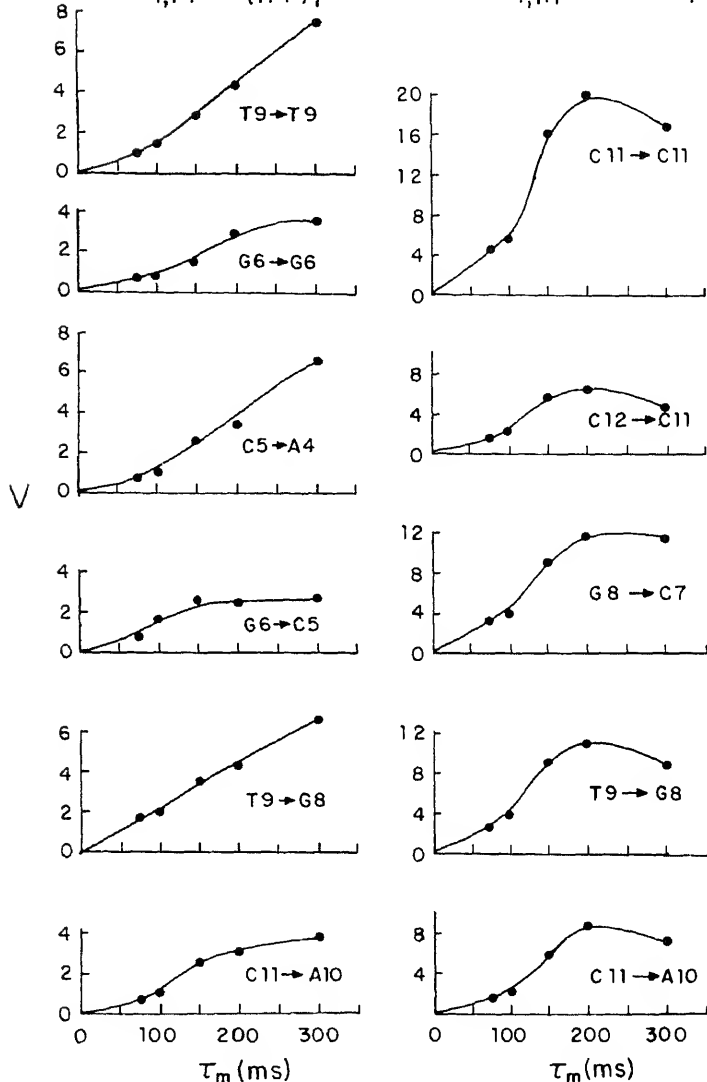
Figure 6. Volumes of a few NOESY cross peaks, in arbitrary units, plotted as a funct[ion of] mixing time for the oligonucleotide d-GGTACGCGTACC.

for G12 (Sheth *et al.*, 1987a), since in these peaks the separation between the far[thest] components yields the sum $J(1'2') + J(2'2'') + J(2'3')$. Extraction of $J(3'4')$ is [more] difficult because of the extreme complexity of H3'-H4' cross peak structure. How[ever] a rough estimate of it can be obtained from the relative intensities of the var[ious] peaks in a low resolution COSY spectrum as discussed elsewhere (Sheth *e[t al.,* 1987b). Such an approach has yielded......information.............

```
5 - 1    2    3    4    5    6    7    8    9   10   11  12 - 3
d - G    G    T    A    C    G    C    G    T    A    C    C
```

(a) ○──3·8──○──3·8──○──3·5──○──3·8──○──3·8──○──3·4──○──3·7──○──3·5──○──3·4──○──4·0──○──4·3──○

(b) ○   ○──3·3──○──3·6──○   ○──3·4──○──3·5──○──3·4──○──3·4──○──3·7──○──3·6──○──3·6──○

(c) ○   ○──3·2──○──3·2──○──3·0──○──3·0──○──2·9──○──3·4──○──3·0──○──3·4──○──3·2──○──3·6──○

(d) ○   ○──3·2──○   ○──3·5──○   ○──4·0──○   ○──3·3──○   ○──3·5──○   ○
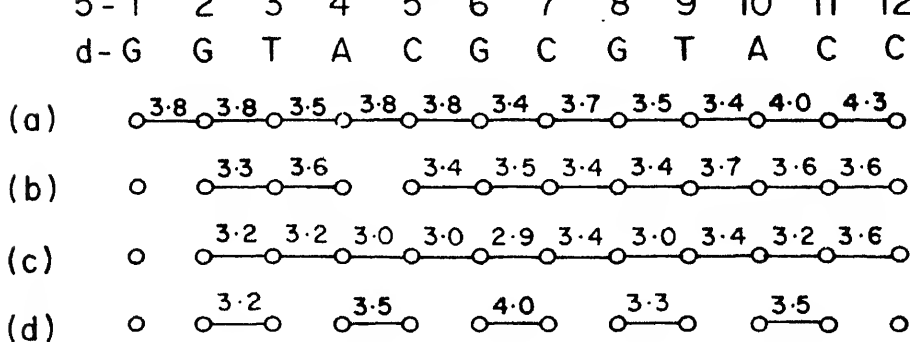
Figure 7. Distances between protons on adjacent nucleotide units, estimated from the volumes of the peaks according to the procedure described in the text. TH_6-TCH_3 distance of 3 Å has been used for reference a, b, c and d refer to the distances $(H8/H6)_n$--$(H1')_{n-1}$, $(H8/H6)_n$-$(H2')_{n-1}$, $(H8/H6)_n$-$(H2'')_{n-1}$ and $(H8/H6)_n$-$(H8/H6)_{n\pm1}$, respectively. Mixing time used in the NOESY experiment was 75 ms.
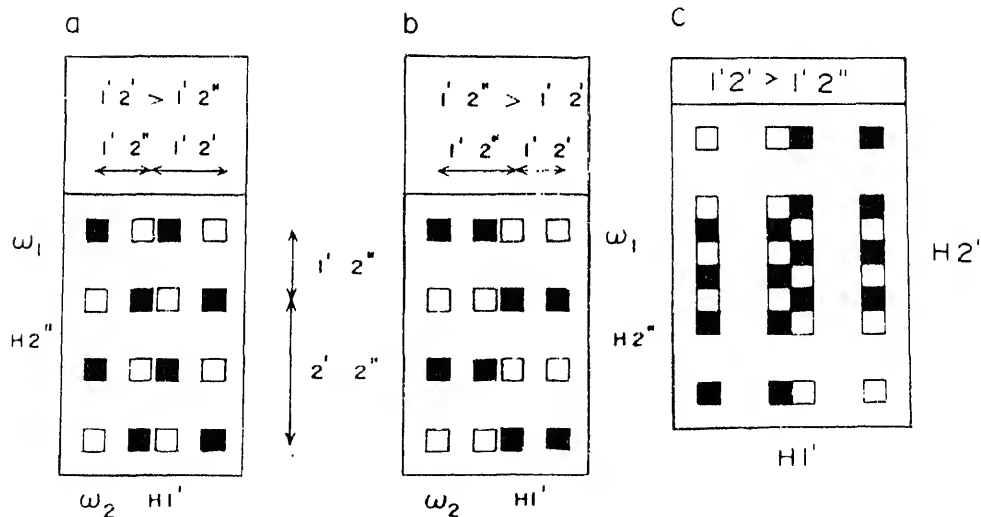
Figure 8. Illustrative cross peak multiplet patterns in COSY spectra of oligonucleotides. The patterns depend on the relative magnitudes of the coupling constants. Filled and open squares refer to positive and negative peaks.

Computer simulation of NOESY spectra

The NOESY spectrum as a whole contains the totality of information about all the short interproton distances within the molecule. However, as must be evident from the preceding discussion, only a limited number of these distances can be measured from the experimental spectrum, since the cross peaks which overlap with other cross peaks cannot be used to estimate the distances. Under such conditions, the obvious

a

ω_1 - Scaled   COSY - 45

d - CTCGAGCTCGAG

$1\cdot8$

ω_1

$2\cdot2$

$(H2', H2$

$2\cdot6$

A

B

$3\cdot0$

$6\cdot2$ $6\cdot0$ $5\cdot8$ $5\cdot6$ $5\cdot4$

ω_2

H1'

b

A
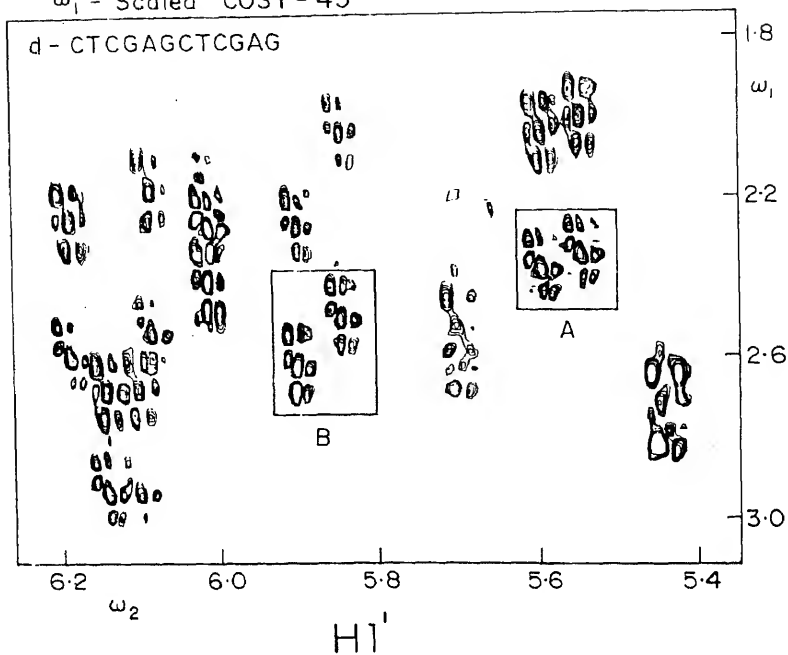
B
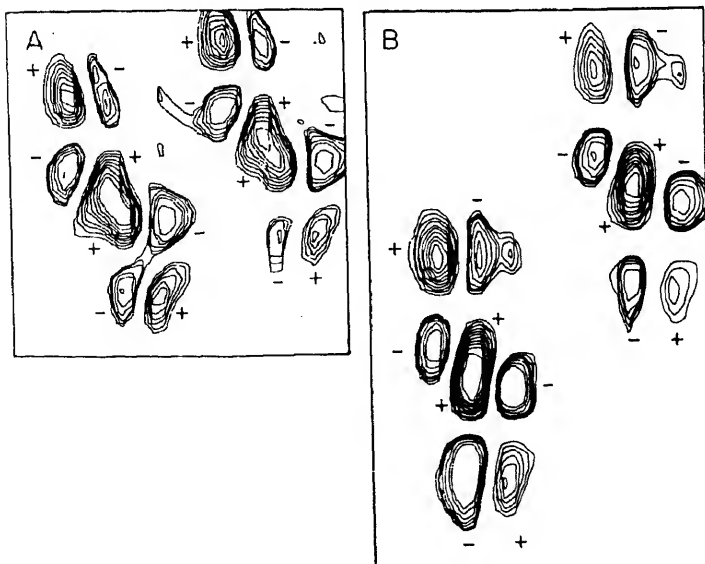
matches with the experimental spectrum in all the regions. Figure 10 shows the flow chart of the simulation procedure (Sheth *et al.*, 1987b), which includes a substantial amount of molecular modelling in an interactive fashion. It is obvious that the number of structures that can be generated is enormous, because of the large number of dihedral angles which can be varied. However, the intranucleotide distance estimates and the individual sugar ring geometries obtained from coupling constant data impose several constraints on the conformational space to be scanned. Besides, the overall pattern of the cross peaks in the NOESY spectrum also provides substantial constraints on the backbone dihedral angles, and these can be used to broadly classify the structure as belonging to the known families of DNA conformations. As illustrated in figure 11, the right- and left-handed structures have distinctly different
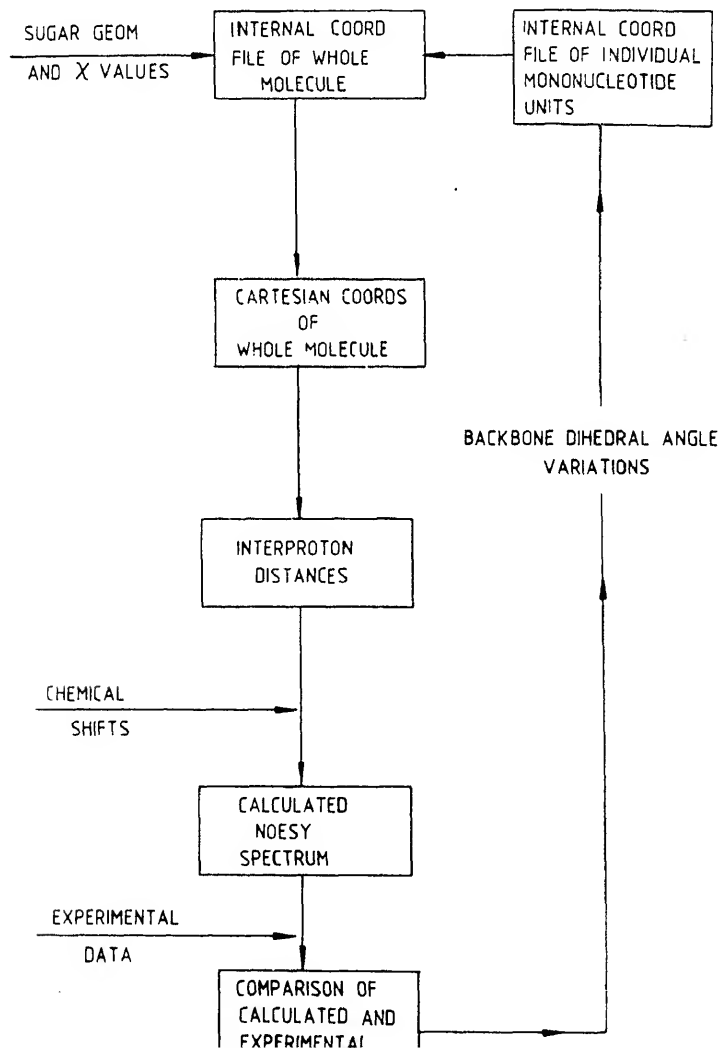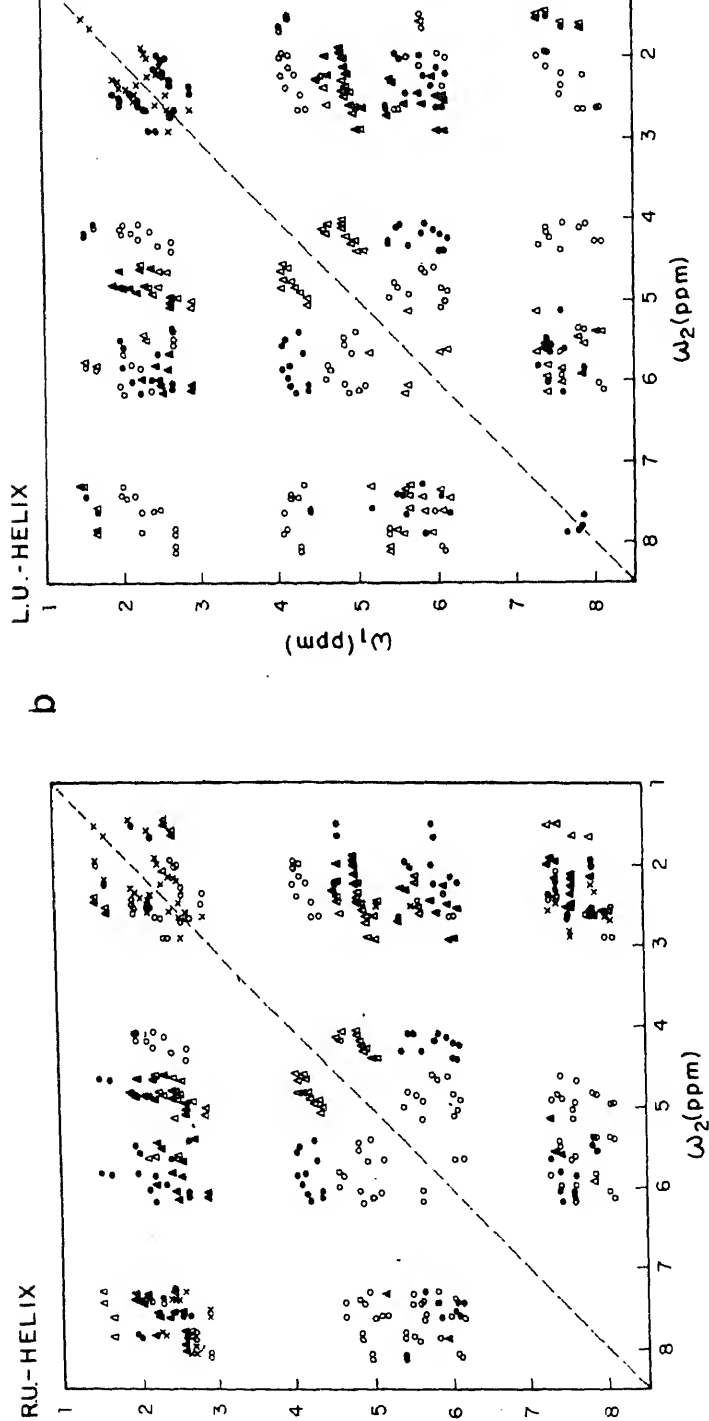
Figure 11. Simulated spectra using the assignments of d-CTCGAGCTCGAG for two standard DNA geometries. The patterns of the cross peaks can be used to identify the overall family of DNA structure from the experimental NOESY spectrum. The different symbols represent different interproton distances (d) and thus indicate the relative intensities of the cross peaks. (X), $1\cdot5 < d < 2\cdot0$; ($\circ$), $2\cdot0 < d < 2\cdot5$; ($\triangle$), $2\cdot5 < d < 3\cdot0$; ($\bullet$), $3\cdot0 < d < 3\cdot5$; ($\circ$), $3\cdot5 < d < 4\cdot0$.

patterns. This of course does not mean that a completely new structure cannot be identified. Detailed analysis of several oligonucleotides using this approach and energy considerations is in progress in our laboratory.

From spectroscopy to molecular structure

Two dimensional NMR spectroscopy has taken us a long way towards determination of molecular structures of nucleic acids in solution. Sequence-specific resonance labels can be obtained for the various protons; interproton distances within the same nucleotide and between adjacent nucleotide units can be measured; spin-spin coupling constants can be measured. These basic inputs are useful in determining the various structural parameters such as helicity, extent of base pairing, sugar geometries, glycosidic dihedral angles and base stacking in the DNA segment. With this knowledge, computer simulation procedures can help in fixing the backbone dihedral angles, using the concept that the NOESY spectrum is a fingerprint of the DNA structure. Finally, energy minimisation can help in further refining the structure.

Acknowledgement

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Biochemical and immunological aspects of riboflavin carrier protein*

P. R. ADIGA†, S. S. VISWESWARIAH., A. KARANDE and N. KUZHANDHAIVELU

Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India

Abstract. Riboflavin carrier protein which is obligatorily involved in yolk deposition of the vitamin in the chicken egg, is a unique glycophosphoprotein present in both the yolk and white compartments. The yolk and egg white proteins are products of a single estrogen-inducible gene expressed in the liver and the oviduct respectively of egg laying birds. Despite the fact that the carbohydrate composition of the yolk and white riboflavin carrier proteins differ presumably due to differential post-translational modification, the proteins are immunologically similar and have identical amino acid sequence (including a cluster of 8 phosphoser residues towards the C-terminus) except at the carboxy terminus where the yolk riboflavin carrier protein lacks 13 amino acids as a consequence of proteolytic cleavage during uptake by oocytes. The protein is highly conserved throughout evolution all the way to humans in terms of gross molecular characteristics such as molecular weight and isoelectric point, and in immunological properties, preferential affinity for free riboflavin and estrogen inducibility at the biosynthetic locus *viz.*, liver. Obligatory involvement of the mammalian riboflavin carrier protein in transplacental flavin transport to subserve fetal vitamin nutrition during gestation is revealed by experiments using pregnant rodent or sub-human primate models wherein immunoneutralisation of endogenous maternal riboflavin carrier protein results in fetal wastage followed by pregnancy termination due to selective yet drastic curtailment of vitamin efflux into the fetoplacental unit. Using monoclonal antibodies to chicken riboflavin carrier protein, it could be shown that all the major epitopes of the avian riboflavin carrier protein are highly conserved throughout evolution although the relative affinities of some of the epitopes for different monoclonal antibodies have undergone progressive changes during evolution. Using these monoclonal antibodies, an attempt is being made to map the different epitopes on the riboflavin carrier protein molecule with a view to delineate the immunodominant regions of the vitamin carrier to understand its structure-immunogenicity relationship.

Keywords. Riboflavin carrier protein; evolutionary conservation; transplacental transport; immunoneutralisation; monoclonal antibodies; epitope analysis.

Introduction

Vitamin carrier proteins capable of high-affinity interaction with their respective vitamins are present throughout the animal kingdom and play a vital role in the life processes of the vertebrates. A great deal of information has been accumulated during the last few years regarding the biological significance of these specific proteins, whose functions include storage and transport of vitamins and prevention of rapid losses of these vital nutrients due to excretion and/or metabolic degradation. Vitamins are known to remain biologically inert as long as they are associated with

biochemical and physiological roles as well as their evolutionary conservation.

Riboflavin carrier proteins

Specific carrier proteins have been identified in oviparous species for riboflavin (Rhodes *et al.*, 1959; Ostrowski *et al.*, 1962), thiamin (Muniyappa and Adiga, 1979, 1981), biotin (Eakin *et al.*, 1940; White *et al.*, 1976), vitamin B_{12} (Sonneborn and Hensen, 1970), retinol (Kanai *et al.*, 1968), vitamin D (Fraser and Emtage, 1976) and folic acid (Krishnamurthy, 1984). It is appropriate to mention that some of these vitamin carriers (for retinol, vitamin D and folic acid) are constitutive to the species, and their hepatic elaboration may be significantly enhanced by appropriate endocrine stimuli to meet the accelerated demand during egg laying. In contrast, others such as those specific for riboflavin, thiamin and biotin are induced *de novo* solely as a reproductive strategem to facilitate vitamin deposition in the developing oocyte (Murthy and Adiga, 1978a; Malathy and Adiga, 1985). These then become detectable both in the eggs and in the maternal circulation. All these vitamins are present at 5–10-fold higher concentrations in the egg than in the maternal circulation; the interaction with carrier proteins apparently facilitates concentration of the vitamins for deposition in the egg (Coates, 1971). These carrier proteins bind the vitamin with a higher affinity than the respective co-enzyme derivatives and this may be a mechanism by which the developing oocyte can sequester the vitamins for its own use in the most appropriate form.

Among the various vitamin carriers hitherto studied from different avian species, chicken riboflavin carrier protein (RCP) is the best characterised, apparently because relatively simple procedures are involved in its isolation in good yields from egg white. Other attractive features associated with this vitamin carrier are: (i) its presence, unlike other major egg proteins, in both egg yolk and white, which could mean dual loci of biosynthesis *viz.*, the liver and the oviduct respectively of egg laying hens, (ii) its inducibility *de novo* presumably by sex steroids and hence its potential as a model system for studies of steroid hormone induced gene expression and (iii) its reversible, high-affinity interaction with flavin which makes it an attractive flavo-protein system with which to understand the structural features involved in ligand-protein interaction. Investigations in our laboratory and elsewhere have led to an understanding of its structure and function in well-defined terms and an overview of the available information is presented below.

RCPs have been isolated from both compartments of the egg (Ostrowski *et al.*, 1962; Murthy and Adiga, 1977) as well as from the serum of laying hens (Murthy and Adiga, 1978b) and the proteins are identical immunologically and biochemically in terms of their affinity and preference for riboflavin. Egg white RCP has recently been crystallised (Zanotti *et al.*, 1984).

Biochemical aspects

Chicken RCP is a phosphoglycoprotein with molecular weight (M_r) 37,000 and

959). Both egg white and yolk RCP were earlier postulated to consist of two identical subunits (M_r 24,000 and 8,000) joined by two interchain disulphide bridges (Philips, 1969). However, this has not been confirmed either in our laboratory or elsewhere (Murthy and Adiga, 1977; Becvar and Palmer, 1982; Kozik,). Analysis of carbohydrate composition of egg white RCP has revealed that the protein contains 10% hexosamine and 4% neutral sugars with a single sialic acid residue at the terminus of a highly branched oligosaccharide chain (Philips, 1969). Yolk RCP contains both hexose and hexosamine as well as multiple sialic acid residues (Ostrowski et al., 1968). The sequences of these oligosaccharides are yet to be elucidated.

Analysis of the amino acid composition of RCP shows the presence of all the common amino acids (Norioka et al., 1985); the protein is particularly rich in glutamic acid, serine and aromatic amino acids. The amino acid sequences of RCP (Figure 1) from egg white and serum have been compared (Norioka et al., 1985) and found to be identical; the yolk RCP also has identical sequence except at the carboxyl terminus where it lacks 13 amino acids. It is therefore reasonable to


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                                10          Lys                    20
<Glu-Gln-Tyr-Gly-Cys-Leu-Glu-Gly-Asp-Thr-His-Lys-Ala-   Pro-Ser-Pro-Glu-Pro-Asn-
                                            Asn
                        30                  CHO              40
Met-His-Glu-Cys-Thr-Leu-Tyr-Ser-Glu-Ser-Ser-Cys-Cys-Tyr-Ala- Asn -Phe-Thr-Glu-Gln-

                        50                              60
Leu-Ala-His-Ser-Pro-Ile-Ile-Lys-Val-Ser-Asn-Ser-Tyr-Trp-Asn-Arg-Cys-Gly-Gln-Leu-

                        70                              80
Ser-Lys-Ser-Cys-Glu-Asp-Phe-Thr-Lys-Lys-Ile-Glu-Cys-Phe-Tyr-Arg-Cys-Ser-Pro-His-

                        90                              100
Ala-Ala-Arg-Trp-Ile-Asp-Pro-Arg-Tyr-Thr-Ala-Ala-Ile-Gln-Ser-Val-Pro-Leu-Cys-Gln-

                        110                             120
Ser-Phe-Cys-Asp-Asp-Trp-Tyr-Glu-Ala-Cys-Lys-Asp-Asp-Ser-Ile-Cys-Ala-His-Asn-Trp-

                        130                             140
Leu-Thr-Asp-Trp-Glu-Arg-Asp-Glu-Ser-Gly-Glu-Asn-His-Cys-Lys-Ser-Lys-Cys-Val-Pro-

                CHO         150                             160
Tyr-Ser-Glu-Met-Tyr-Ala- Asn -Gly-Thr-Asp-Met-Cys-Gln-Ser-Met-Trp-Gly-Glu-Ser-Phe-

                        170                             180
Lys-Val-Ser-Glu-Ser-Ser-Cys-Leu-Cys-Leu-Gln-Met-Asn-Lys-Lys-Asp-Met-Val-Ala- Ile -

                        190                             200
Lys-His-Leu-Leu-Ser-Glu-Ser-Ser-Glu-Glu-Ser-Ser-Ser-Met-Ser-Ser-Ser-Glu-Glu-His-
                         |   |       |   |   |       |   |   |
                         P   P       P   P   P       P   P   P
                        210                             219
Ala-Cys-Gln-Lys-Lys-Leu Leu-Lys-Phe-Glu-Ala-Leu-Gln-Gln-Glu-Glu-Gly-Glu-Glu.
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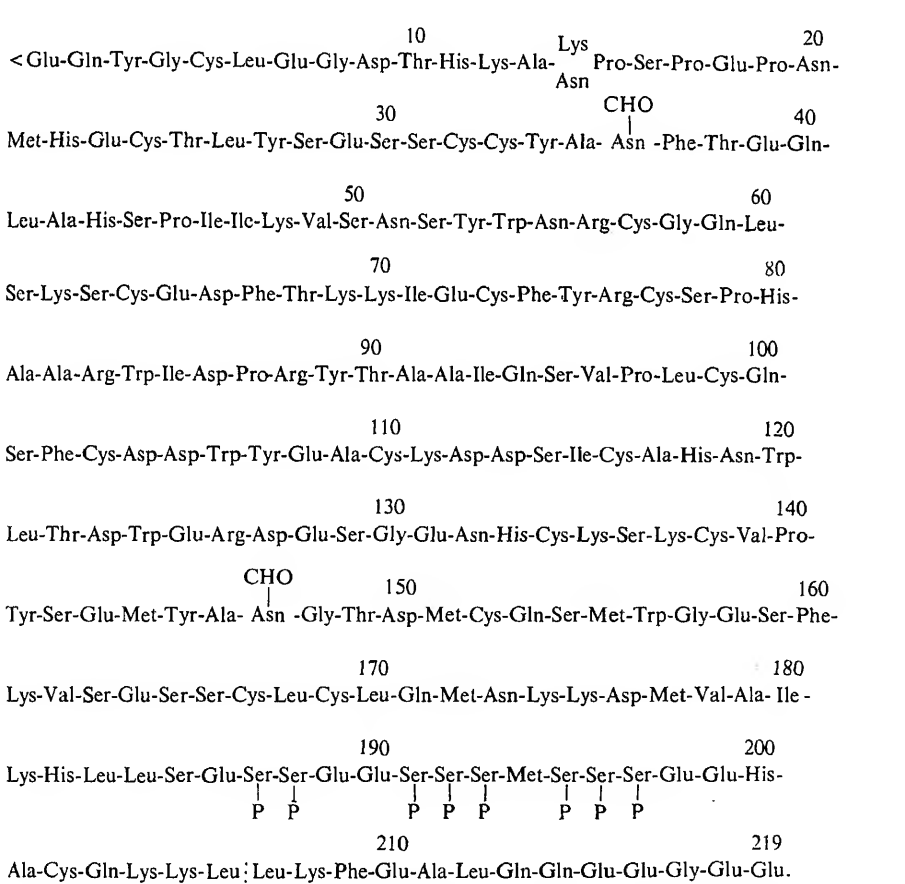
Figure 1. Amino acid sequence of chicken egg white riboflavin carrier protein (from

(Bergink *et al.*, 1974). Thus RCP is the second example among yolk proteins that is enzymatically modified during incorporation into the yolk. In other respects, yolk RCP has the same characteristics as egg white RCP, and these include the N-terminal pyroglutamic acid, polymorphism in the amino acid sequence (lysine/asparagine at the 14th residue from the N-terminus) and carbohydrate chains attached to Asn-36 and Asn-147 residues. Phosphate groups are also bound to the same serine residues which occur in a cluster between positions 187 and 197 in both yolk and white RCPs. All these observations confirm the earlier hypothesis based on genetic analysis that yolk, plasma and white RCPs are derived from the same structural gene (Norioka *et al.*, 1985).

An intriguing aspect of this phosphoprotein is that all the phosphate groups are localised as phosphoserine moieties in a restricted, highly anionic region of the peptide chain (Norioka *et al.*, 1985); thus within a 21 amino acid segment are found 8 phosphoserine residues and 5 glutamate residues (figure 1). This peptide can be isolated by trypsin digestion of reduced and carboxymethylated RCP (Miller *et al.*, 1984) and has lysine at its C-terminal and histidine at the N-terminal ends. Sandwiched between these two basic amino acids is a sequence of 21 amino acids among which 14 carry one or two negative charges at physiological pH. In view of the high degree of charge repulsion, it is assumed that this phosphopeptide is rigid, with little or no ordered secondary structure. A highly conspicuous feature of this phosphopeptide is the palindromic sequence around Met-194. This residue is sandwiched between 6 phosphoserine and 4 glutamic acid residues in a defined sequence. The biological significance of this rather unique amino acid sequence is currently unknown.

The carbohydrate composition of yolk RCP is identical to that of plasma RCP, but both differ from that of egg white RCP showing that the post-translational attachment of carbohydrate chains of RCP differs in the liver and oviduct. However, it is intriguing that the phosphorylation sites of egg yolk RCP are similar to those of egg white RCP, indicating that protein kinases with similar specificities participate in the phosphorylation of the protein in the liver and oviduct (Norioka *et al.*, 1985).

Riboflavin binding characteristics of chicken RCP

Every region of RCP has been investigated for flavin binding, receptor recognition as well as antigenicity. Extensive work has been carried out on the flavin binding characteristics of the protein in an attempt to understand the sites of flavin-protein interactions apparently as a model for flavin co-enzyme-enzyme interaction. The apoprotein binds to a variety of flavin analogues in a 1:1 stoichiometry and shows a preferential affinity for riboflavin (Nishikimi and Kyogoku, 1973). The fluorescence of flavin is completely quenched on binding to egg white apo-RCP while 80% of the protein fluorescence is quenched on binding to riboflavin and 3-methyl riboflavin and 77% on binding to lumiflavin (Nishikimi and Kyogoku, 1973). Nuclear magnetic resonance data indicate that the chemical shifts of the carbon and nitrogen atoms of riboflavin hardly differ on binding to either egg white or yolk RCP indicating that the binding site for the oxidised isoalloxazine ring are similar in the two isoproteins

Moreover, N-3 of riboflavin is exposed to the solvent while N-10 and ribityl side chain are strongly involved in the interaction with the protein by formation of hydrogen bonds (Matsui *et al.*, 1982b; Moonen *et al.*, 1984). Studies using model compounds with different modifications of the flavin molecule reveal that the dimethyl benzenoid part of the ring is the predominant portion involved in interaction with the apoprotein and gets buried to a large extent in the protein (Choi and McCormick, 1980).

The flavin binding site in the protein has been studied by chemical modification of the protein. From the data obtained hitherto, it appears that tryptophan residues are essential for the binding of riboflavin (Murthy *et al.*, 1976; Blankenhorn, 1978). Modification of 5 tryptophans completely abolished the flavin binding ability of yolk RCP (Miller *et al.*, 1981a) and it has been proposed earlier that 1–2 tryptophans are essential for riboflavin binding in egg white RCP (Murthy *et al.*, 1976). More recent studies have however revealed that one of these tryptophan residues is critically involved in the binding of flavin and this tryptophan is not protected by bound flavin against chemical modification. Tyrosine is not critically involved in flavin binding because extensive iodination or nitration does not alter the flavin binding capacity (Farrell *et al.*, 1969). However, a further study has established that one tyrosine is apparently located at the binding site since it is protected against chemical modification by bound flavin (Blankenhorn, 1978).

Earlier work (Murthy, 1977) has indicated that at a low pH, the protein undergoes self-aggregation leading to a reduced affinity for the vitamin. In fact, there is nearly 100-fold reduction in riboflavin binding capacity on lowering the pH from 7·4–4. The presence of sodium dodecyl sulphate (SDS) also reduces the binding. However, interaction of the protein with polyclonal antibody or concanavalin-A does not seem to change its affinity for riboflavin, suggesting that the riboflavin binding site is distinct from antigenic sites (Murthy, 1977).

Receptor recognition sites on RCP

The function of RCP, as mentioned earlier, is confined to the deposition of the vitamin in the developing oocyte. It is also believed that RCP, mostly present in apoprotein form in egg white, may have a bacteriostatic role in sequestering the free vitamin released into the albumen during embryonic development (Board and Fuller, 1974). On the other hand, RCP present in the yolk is involved essentially in meeting the nutritional requirements of the growing embryo. Yolk RCP is deposited into the yolk from the blood, with the vitamin firmly bound to it; direct evidence for this stems from experiments using mutant hens afflicted with the hereditary syndrome avian riboflavinuria (Maw, 1954). When RCP isolated from the eggs of normal hens was injected into laying hens homozygous for the avian riboflavinuria trait, and the eggs examined for evidence of transfer of RCP by immunoprecipitation, the protein was detected at low levels in the egg yolk at 2 days following injection, but none was found in the egg white (Hammer *et al.*, 1971). This shows clearly that the blood protein is directly incorporated into the yolk. Furthermore, removal of sialic acid

of galactose and removal of N-acetylglucosamine and galactose also led to diminished transport of the protein into the yolk (Miller *et al.*, 1981b) despite the fact that the protein still retained riboflavin binding activity. A comparison of the carbohydrate composition of yolk and circulatory RCPs indicates removal, during ovarian uptake, of one sialic acid, one fucose, two galactose, and 3 N-acetylglucosamine residues from the precursor serum RCP. However, it may be pointed out, despite the obvious implication that the carbohydrate (especially the sialic acid) residues are involved in uptake by the oocyte, that no direct evidence for binding of RCP to the ovarian follicular membrane has been demonstrated so far (Miller *et al.*, 1982a).

Another region of RCP which has been implicated in oocyte membrane recognition is the phosphopeptide moiety; dephosphorylation of egg white RCP or yolk RCP has no effect on the binding of riboflavin by the protein, but oocyte uptake of the dephosphorylated protein is greatly reduced (Miller *et al.*, 1982b). Removal of a single phosphate residue from yolk RCP decreases follicular uptake by 60% and this cannot be restored by the addition of anionic groups such as by succinylation (Miller *et al.*, 1982b). The phosphopeptide portion appears to function autonomously of the rest of the protein and could be involved in recognition of the putative receptor on the oocyte membrane, either through direct interaction or by directing the protein in such a way as to facilitate subsequent interaction with the receptor in a potential gradient (Miller *et al.*, 1984).

Interestingly, succinylation of the native protein also decreases its uptake by the oocytes (Miller *et al.*, 1981b) indicating that uptake also involves other segments of the peptide chain. Hence, uptake of RCP by oocytes may be a complex sequence of protein-receptor interaction involving phosphate, sialic acid and lysine residues and elucidation of the mechanism awaits further research.

Mammalian RCPs

In contrast to the extensive knowledge available on chicken RCP, the information on mammalian RCPs is limited to a few cases. The first demonstration of RCP from a mammalian source was reported from our laboratory (Adiga and Muniyappa, 1978; Nutrition Reviews, 1979). Using a sensitive radioimmunoassay (RIA) involving iodinated chicken RCP and antiserum to chicken RCP, a protein cross-reacting with chicken RCP could be detected in pregnant rat serum (Muniyappa, 1980). The protein has been purified by lumiflavin-affinity chromatography, though its M_r was ambiguous. More recent data from our laboratory however reveal that the rodent RCP purified by fast protein liquid chromatography has a molecular size comparable to that of chicken RCP (Karande, A. A. and Adiga, P. R. unpublished results). Evidence for the functional role of rat RCP has also been obtained. Administration of antibodies to chicken RCP to pregnant rats leads to pregnancy termination (Muniyappa and Adiga, 1980) consequent to a decrease in uptake of riboflavin by the developing embryo (Murty and Adiga, 1981). Further studies on the

wing active immunisation of normal female rats with chicken RCP the
ient chronic *in vivo* immunoneutralisation of maternal RCP leads to
tion of pregnancy around days 8–10 of gestation. It has been proposed that
P may not be involved in the fertilization/implantation process *per se* but is
ly required for providing adequate riboflavin to the developing fetus (Murty
iga, 1982a). Pregnancy continues to term with the delivery of normal pups
rculating antibody concentrations are allowed to wane with time in actively
sed rats (Murty and Adiga, 1982a), showing the reversibility of the immuno-
sation process.

her claim for the occurrence of RCP in a mammal was made by Merrill *et al.*
who purified RCPs from bovine plasma and adduced evidence for a
icy-specific riboflavin binding protein. These proteins have been isolated by
chromatography using N^3-carboxymethyl riboflavin coupled to AH-
se. At least 3 major protein bands could be observed migrating in regions
d to the β- and γ-globulins of plasma following cellulose acetate electro-
s. The M_r of one of these proteins was 150,000 as assessed by gel filtration,
interesting observation was that a small amount of another riboflavin
protein of M_r 37,000 was also present. All 3 proteins bound $[^{14}C]$-riboflavin
with high affinity (kd $= 10^{-6}$ mol/litre). The presumed pregnancy-specific, low
tein from bovine serum was purified to apparent homogeneity and appeared
an even higher affinity for riboflavin. No further analysis of these proteins
n forthcoming, but it is claimed that a certain protein binding riboflavin with
finity is associated with pregnancy in higher mammals with a function
us to that of serum RCP in laying hens.

er studies on RCPs in higher mammals were confined to their detection and
characterisation in human sera. Merrill *et al.* (1979) have reported that
albumin, which is known to associate with riboflavin with low affinity (Jusko
vy, 1975), a certain fraction of immunoglobulin G (IgG) also binds riboflavin
reasonably high affinity (4 μM) (Merrill *et al*., 1981). This fraction (about 1%
IgG) could be isolated by affinity chromatography and is non-specific in the
iat it is present in the sera of male and female non-pregnant and pregnant
uals. However, because of the relatively higher concentrations of albumin in
ım it has been suggested that only 5–6% of protein-bound riboflavin in
human plasma is associated with this IgG fraction (Merrill *et al.*, 1981).

ms unlikely that riboflavin is the antigen inducing these immunoglobulins
conceivable that ligand binding is accomplished on a site on these proteins
accomodates the flavin and/or ribityl side-chains. Eisen *et al.* (1970) have
d that a monoclonal immunoglobulin A produced by mouse plasmacytoma
-315 binds riboflavin (Kd 36 μM) and other hydrophobic compounds
tely tightly. A much tighter binding of riboflavin has been reported by
gi and Osserman (1976) for a monoclonal IgG (IgG^{gar}) produced by a patient
ultiple myeloma. This human monoclonal IgG 2 (λ) is separable into two
fractions by ion-exchange chromatography; one of these fractions is nearly
d with the ligand with an average of about 2 equivalents of riboflavin/mol,

al., 1981a, b). Using a variety of flavin analogues, the regions on the riboflavin molecule which associate with the binding site have been identified (Pologe *et al.* 1982). The pyrimidine edge of the isoalloxazine does not interact with the combining site, particularly around N-3. The ribityl side chain and dimethyl benzene edge of the flavin ring are critical for binding and are probably not exposed (Pologe *et al.*, 1982). A comparison with flavoproteins show that IgG^{gar} binds riboflavin in a rather novel way. The isoalloxazine ring interacts in a way similar to that in FMN binding proteins, but unlike the situation in flavoproteins, the ribityl side chain is not essential for binding. Further investigations are required to shed light on the essential features of this flavin binding site and the way in which such a site is manifest as an integral part of a human monoclonal immunoglobulin.

There has been a recent claim that another protein fraction which binds riboflavin with high affinity can be obtained from human fetal cord blood and is present in relatively smaller amounts (25 μg/15 ml blood) (Merrill *et al.*, 1981). Further information regarding molecular size or other characteristics is not available at present, but it has been proposed that human blood like blood of other mammals, contains proteins which may serve an ancilliary role to albumin in pregnancy, analogous to the role of RCP in avian eggs.

We have recently isolated and characterised RCPs from pregnant bonnet monkey and human sera (Visweswariah and Adiga, 1987a, b). A heterologous RIA using [^{125}I]-labelled chicken RCP and antiserum to chicken RCP was employed to show that sera from pregnant bonnet monkeys and humans (Visweswariah and Adiga, 1987a, b) are able to inhibit the binding of chicken RCP to specific antiserum. Human umbilical cord serum also contains a cross-reacting protein, in higher concentrations than in maternal pregnancy sera. Isolation of these proteins involves sophisticated protein purification techniques such as fast protein liquid chromatography involving ion-exchange and chromatofocusing. The purified proteins exhibited properties with remarkable similarities to those of the chicken vitamin carrier. Thus, the M_r of both monkey and human RCP (from either pregnancy or umbilical cord sera) are similar to that of chicken RCP (37,000). All the proteins are acidic in nature (pI < 4) and preferentially bind riboflavin *vis-a-vis* FMN and FAD. The purified proteins bind to specific antibodies against chicken RCP which is indicative of extensive sequence similarity amongst the proteins. The sequence similarity could be confirmed by comparing the tryptic peptide maps of [^{125}I]-labelled monkey RCP and chicken RCP. Thus, RCP is a protein which has been retained to near identity from aves to primates. This is strongly suggestive of a vital role for this protein in primate reproduction as well.

Hormonal modulation of RCP

It is now well established that the *de novo* synthesis of yolk proteins in the liver of

chicks. The induced proteins are secreted by the respective biosynthetic organs even in the absence of a developing oocyte in which they are normally sequestered (Cecchini *et al.*, 1979) and hence plasma or oviduct tissue concentrations reflect the synthetic capacities of liver and oviduct, respectively.

We developed (Murthy and Adiga 1978a) a RIA for chicken RCP to monitor the circulating levels of the protein following estradiol-17β administration to immature male chickens. After a single injection of the hormone, the plasma RCP level is enhanced several-fold at 6 h, reaching peak levels around 48 h and declining thereafter to the basal level. A 2-fold amplification of the response is observed on secondary stimulation with the hormone. A 4 h lag phase prior to the onset of induction is noticed during both primary and secondary stimulations with the hormone. The synthesis of the protein is dependent on the dose of hormone administered, with the maximum effect observed with 10 mg/kg body weight. There is no appreciable change in the half-life of the protein on estradiol-17β administration but the half-life is modulated by the thyroid status of the animal (Murthy and Adiga, 1978a). Progesterone is unable to affect the kinetics of estradiol-17β induced RCP production, but antiestrogens are potently capable of blocking the response.

A detailed comparison of the induction of RCP in the liver and oviduct has been carried out in our laboratory (Durga Kumari, 1984). On primary stimulation of immature female birds with estradiol-17β (10 daily injections), there is a rapid increase in oviduct weight and total RCP after an initial lag period of 2–3 days. Secondary stimulation with estradiol-17β results in a rapid increase in RCP levels without the lag period. Progesterone treatment results only in a slight increase in oviduct weight; it can also activate the differentiated oviduct cell function in terms of RCP synthesis, but only during secondary stimulation, *i.e.*, after primary stimulation with estradiol-17β. The plasma levels of RCP in these birds reflect progressive increase in synthesis of RCP on primary stimulation with estradiol-17β and the characteristic memory effect with attendant amplification of the inductive response during secondary stimulation. However, progesterone is unable to stimulate the synthesis of RCP by the liver when administered as secondary inducer, unlike the phenomenon observed in the oviduct (Durga Kumari and Adiga, 1986). These observations bring into focus subtle qualitative differences in the hormonal regulation of the RCP gene in the two estradiol-17β dependent avian tissues. The differences may be a reflection of differential modulation of tissue-dependent regulatory elements governing RCP gene expression in the two biosynthetic loci.

Cell-free translation of (polyA$^+$) RNA from both liver and oviduct has revealed that enhanced RCP mRNA levels account for the increased synthesis of RCP in these two tissues (Durga Kumari, 1984). In a heterologous cell-free translation system, *viz.*, rabbit reticulocyte lysate, a precursor RCP of M_r 38,000 is identified which is processed to native RCP in the presence of stripped microsomes from avian liver. The increased mRNA activity associated with chicken RCP production on secondary stimulation could be correlated with greater number of mRNA molecules due to enhanced transcription and/or due to stabilization of cytoplasmic RCP mRNA during secondary stimulation, as shown for vitellogenin, ovalbumin and

administration of estradiol-17β (Muniyappa and Adiga, 1980). Moreover, the levels of the protein is clearly modulated by circulatory concentrations of estrogen during the 4-day estrous cycle: the concentration of the protein is highest during pro-estrous when estrogen concentration is the highest. The concentration of the protein appears to change during pregnancy with a gradual increase to peak around day 10 of gestation and is maintained more or less at high levels till term. These studies employed a heterologous RIA utilising [^{125}I]-labelled chicken RCP and antiserum to chicken RCP. Using a homologous RIA for rat RCP, the hormonal induction of the protein has been investigated (Murty and Adiga, 1982b) and the data are essentially in agreement with the results reported earlier. It is noteworthy that the rodent protein is also induced specifically by estradiol-17β in a dose-dependent manner and its synthesis is blocked by cycloheximide (Murty and Adiga, 1982b).

The circulatory concentrations of monkey and human RCPs are also modulated by physiological changes in estrogen concentration that occur during the menstrual cycle and pregnancy. Administration of estradiol-17β to immature male or female monkeys is able to enhance circulatory concentrations of monkey RCP (Adiga *et al.*, 1986; Visweswariah, 1986). These results clearly reveal that the evolutionary conservation of RCPs extends not only to structural and physicochemical properties but also to their estrogen-denpendent elaboration by the liver. This implies an important role for these proteins during gestation in primates, presumably analogous to that established in the avian and rodent models. Confirmatory evidence for this premise stems from recent observations in our laboratory that active immunisation of adult female bonnet monkeys with chicken RCP leads to early termination of pregnancy, provided that the antibody titres in circulation are high enough to neutralise endogenous protein (Adiga *et al.*, 1986). These results lend credence to our working hypothesis that the immunological similarities between the RCPs is a reflection of the vitamin carrier performing a definite and important function in primate reproduction in terms of facilitating transplacental flavin transport from the maternal supply line to the developing fetus (figure 2).
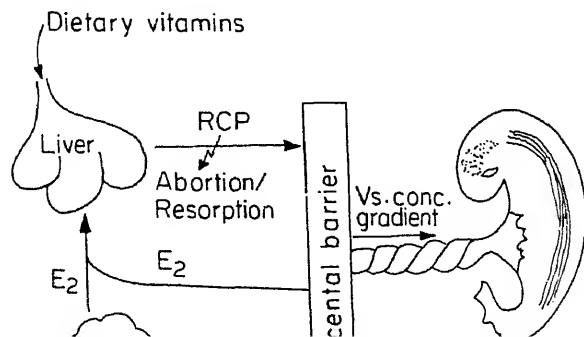
from the aves to humans, not only in terms of similarities in gross structure, but also with regard to the hormonal modulation of its induction during the reproductive phase and its obligatory role as vitamin carrier from the maternal system to the developing oocyte/embryo. It is now well recognised that fixation of changes in protein sequence or structure depend on whether the changes will be compatible with the biochemical function of the protein and the degree of dispensability of the protein for the survival of the organism. The gross similarities among the RCPs throughout evolution therefore emphasise the vital role this protein has to play during reproduction.

Immunological studies on RCP

The similarities in the physicochemical properties of various RCPs extend to the extensive immunological cross-reactivity observed with polyclonal antisera to chicken RCP. A strong, although not perfect, quantitative correlation exists between amino acid sequence similarity and immunological resemblance. Thus, proteins which have a greater than 40% divergence in amino acid sequence, show no immunological cross-reactivity (Arnheim, 1973; Wilson et al., 1977), and in general, the degree of cross-reactivity observed between two homologous globular proteins is directly related to the degree of resemblance between their amino acid sequences (Arnheim, 1973; Wilson et al., 1977). Therefore, by implication, the immunological cross-reactivity observed amongst RCPs is highly suggestive of similarities in amino acid sequences. However, the cross-reactivities observed with whole polyclonal antisera are influenced by a number of variables, such as the relative and absolute concentration and affinities of the different determinant-specific antibodies which comprise the antisera as well as the inevitable variations in response to antigen by individual animals. Monoclonal antibodies (MAbs), however, can provide an immunological comparison of proteins on a determinant-by-determinant basis, since small changes in protein structure may produce large changes in immunological cross-reactivity. Certain MAbs have been known to discern even single amino acid changes in the sequences of two proteins (Harris, 1983) and hence, by virtue of their property of each reacting only with a single determinant, may provide exquisitely sensitive probes for discriminating between structurally related proteins. With this view in mind, we have raised MAbs to chicken RCP in an attempt to study more closely the homology in various determinants between RCPs of different species and in order to gain further insight into the antigenic map of chicken RCP and sequence divergence, if any, in the RCPs present in mammalian sera.

Studies hitherto on the antigenicity and antigenic domains of chicken RCP are few and more detailed analysis is needed. The protein is highly antigenic and antibodies can be raised in a variety of mammalian species, viz., rabbit, rat and monkey (Cotner, 1972; Ramanathan et al., 1979). Chemical modifications of RCP reveal that the protein moiety largely contributes to the antigenicity of the protein (Ramanathan et al., 1980). Total reduction of the disulphides, NBS-oxidation of the tryptophans and succinylation or dinitrophenylation of the lysine residues results in a loss of

retention of flavin binding activity, but the slope of the inhibition curve of the amidated derivative in RIA is different from that of the unmodified RCP indicating a weakening of almost all antigenic determinants (Cotner, 1972; Ramanathan et al., 1980). Modifications of tryptophan and tyrosine residues in the protein do not alter its antigenic properties, but leads to a complete loss of flavin binding properties (Ramanathan et al., 1980). These observations lead to the inevitable conclusion that antigenic sites on the molecule are mostly localized in areas different from the riboflavin binding site. Moreover, holoflavoprotein and apoflavoprotein react similarly in RIA and Ouchterlony immunodiffusion analysis; the apoprotein bound to its antibody on an affinity column still interacts with flavin at 97% of the theoretical amount. The absence of any cross-reacting peptides in the trypsin hydrolysate of the citraconylated, totally reduced and alkylated apoprotein suggests that the antigenic determinants depend on secondary and/or tertiary structure (Murthy and Adiga, 1978a). Lysine residues may be involved either at the actual antigenic sites and/or their modification leads to drastic changes in the conformation of the protein.

Lysine residues of globular proteins are mainly found localized on the surface of the molecule and protrude into the solvent rather than react with other residues (Arnheim, 1973; Wilson et al., 1977). There are several cases reported where the biologically active site is independent of the antigenic site and this is in agreement with the much studied phenomenon of the conservation of the active site of many enzymes through various stages of phylogenetic development (Arnheim, 1973; Wilson et al., 1977). It is attractive to raise the question at this stage whether various RCPs have retained an identical amino acid sequence/tertiary structure at the riboflavin binding region during evolution. With a view to study in greater detail the immunological cross-reactivity amongst avian and mammalian RCPs by a sensitive determinant-by-determinant approach, we have generated MAbs to chicken RCP by employing the hybridoma technique developed by Kohler and Milstein (1975). We have used the myeloma SP2/0-Ag 14 as the fusing partner of mouse splenocytes and optimised immunisation protocols. Mice were immunised with chicken egg white RCP (Visweswariah, 1986). Fusion was performed with 50% polyethylene glycol (PEG 4,500) and 10% dimethyl sulphoxide and the hybrid clones were screened by enzyme linked immunosorbent assay and a solid phase protein A binding assay (Visweswariah et al., 1987). Three MAbs have been extensively characterised and their properties have been described recently (Visweswariah et al., 1987). The affinity calculated by Scatchard analysis for the parent antigen varies as expected with each antibody. These antibodies do not appear to differentiate between holo-and apo-RCP, in agreement with observations with polyclonal antisera. Denaturation of RCP with SDS also does not modify the interaction of the protein with these MAbs, but earlier results from this laboratory have shown that chicken RCP treated with SDS has significantly reduced affinity for riboflavin (Murthy, 1977). This is consistent with the premise that the flavin binding site is distinct from the major antigenic determinants recognised by the 3 MAbs. However, as shown recently (Visweswariah et al., 1987), total denaturation of RCP by reduction and carboxymethylation eliminates recognition of the modified protein by the MAbs. This shows that none

of these MAbs recognize a linear sequence of amino acids *per se* and that at least a partially native conformation of chicken RCP is essential for interaction with these antibodies. Interestingly, succinylated chicken RCP is also not able to inhibit the binding of [^{125}I]-labelled native chicken RCP to these MAbs even at a 100-fold excess concentration, indicating that lysine residues are involved in the recognition of the protein by these antibodies, in agreement with the observations made with polyclonal antisera.

Using a novel method of epitope analysis using Superose 12 gel filtration in conjunction with fast protein liquid chromatography, it could be shown that the 3 MAbs are directed to 3 different and distinct epitopes on the chicken RCP molecule (Karande *et al.*, 1987). Employing these MAbs, studies were initiated to ascertain whether the epitopes on chicken RCP to which these MAbs are directed are conserved in mammalian RCPs. As expected we could indeed observe an inhibition of binding of [^{125}I]-labelled chicken RCP to each of these MAbs by different concentrations of rat, monkey and human RCPs (Visweswariah *et al.*, 1987). This indicates that the epitopes defined by these MAbs are clearly present in mammalian RCPs as well. By employing RCP isolated from human pregnancy serum and umbilical cord serum, we could show that at least in the regions defined by the 3 MAbs, the two proteins were nearly identical, as gauged by very similar affinities of the MAbs for them (Visweswariah, 1986). If one assumes that the protein from cord serum is largely of fetal liver origin, then there is apparently no significant difference between the embryonic and the adult RCP gene products at least in terms of these epitopes. The results obtained using MAbs are in close agreement with our earlier observations employing polyclonal antisera and provide a means of mapping the various epitopes on the chicken RCP molecule.

Towards this end, a number of other MAbs to chicken RCP have been produced and are being characterised at present (Kuzhandhaivelu, N., Karande, A. A. and Adiga P. R., unpublished results). Preliminary results indicate that one of the MAbs is able to recognise egg white RCP but not egg yolk RCP. As stated earlier, the difference between these isoproteins is the two carbohydrate chains and the 13 amino acid chain at the C-terminus which is present in egg white and serum RCP but is cleaved off during uptake by oocytes to produce mature yolk RCP (Norioka *et al.*, 1985). It is therefore likely that one of the MAbs recognises the conformation associated with these 13 amino acids, and this region could represent a continuous epitope on the chicken RCP molecule (Sela, 1969). Alternatively, this MAb could recognise one of the carbohydrate chains exclusive to the egg white. We also have preliminary evidence to show that one of the other MAbs recognizes in solid-phase RIA the phosphopeptide isolated from egg white RCP representing the sequence of amino acids from 181–204 (figure 1).

An attempt has been made to theoretically predict the possible antigenic domains on the chicken RCP molecule by performing a hydrophilic analysis (Hopp and Woods, 1981; Visweswariah, 1986) using the known amino acid sequence of chicken RCP (Norioka *et al.*, 1985). This exercise was prompted by the recent evidence that
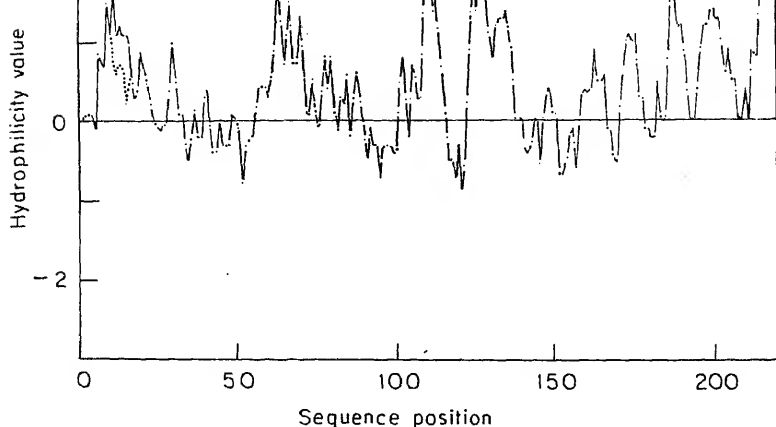
Figure 3. Hydrophilicity profile of chicken RCP. The hatched line indicates the profile obtained when asparagine is present in the sequence at position 14 instead of lysine. The averaged hydrophilicity values are plotted versus position along the amino acid sequence. The x-axis contains 214 increments, each representing an amino acid in the sequence of chicken RCP. The y-axis represents the range of hydrophilicity values from −3 to +3. The data points are plotted at the centre of the averaging group from which they were derived.

hydrophilicity is in sequence (108–118) and this is likely to be an antigenic domain. It appears that there are 3 major hydrophilic regions in the molecule (60–70, 105–115, 120–140) and it is attractive to propose that the MAbs described here are directed to these 3 regions.

This theoretical excercise may be able to predict with a certain degree of confidence some sequences which comprise antigenic domains of the protein. However, the smaller hydrophilic peaks are not always associated with immunogenic sites (Hopp and Woods, 1981). An improved method which eliminates to a great extent the redundancy of prediction makes use of the recognition factors of various amino acids (Fraga, 1982) and this correction has been applied to the analysis of chicken RCP (figure 4). Each amino acid in the chicken RCP primary sequence is assigned a recognition value and these values are repeatedly averaged over 6 residues. Figure 4 indicates the recognition value of the residue at the mid-point of each hexa-peptide. The assumption that regions of high hydrophilicity and low recognition are antigenic may lead to accurate prediction of antigenic domains with a high success rate. It can be seen that some regions of highest hydrophilicity in chicken RCP have a very low recognition value and therefore are most likely to be contained within antigenic determinants. Based on a similar consideration, the region (120–140) is also likely to comprise a determinant. However, other minor peaks of hydrophilicity appear to be non-immunogenic in that they coincide with peaks in the recognition profile. The region of the phosphopeptide (182–204) perhaps is of greatest interest, since a definite biological activity has been assigned to it recently (Miller et al., 1982b). This sequence is contained within a region of high
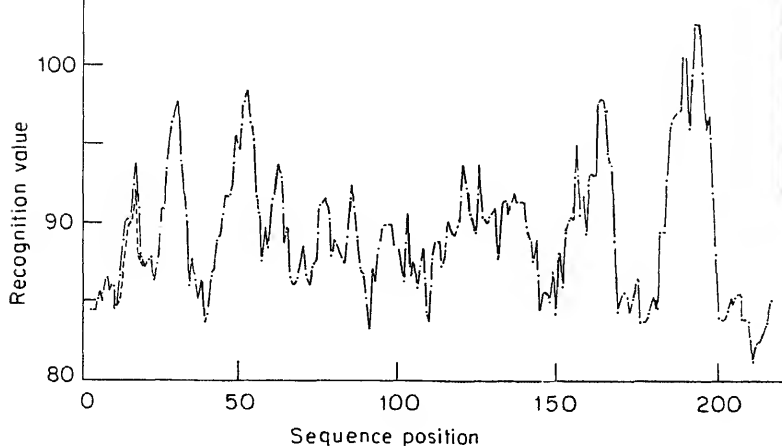
Figure 4. Recognition profile of chicken RCP. Recognition values were assigned to each residue in chicken RCP and repeatedly averaged over each hexapeptide. These averaged values are plotted versus position along the amino acid sequence. The y-axis represents the range of recognition values, and the data points are plotted at the centre of the averaging groups from which they were derived. The hatched line indicates the values obtained when asparagine is present in the sequence at position 14 instead of lysine.

recognition and is normally unlikely to be within an antigenic domain. However, in all these analysis, no correction is made for amino acid residues modified by phosphorylation or glycosylation and it is quite likely that these may cause shifts in both hydrophilicity and recognition value. The high charge of the phosphate in chicken RCP could induce certain changes in the structure of the protein such that the phosphopeptide is exposed to the surface. Isolation of the phosphopeptide and a study of its immunogenicity will provide information on whether this region is contained within an immunodominant site or not.

The hydrophilic analysis in conjuction with the recognition profile of chicken RCP could thus provide information on the possible peptide sequences to which the MAbs to chicken RCP are directed. The analysis also explains certain conclusions reached from experiments conducted with polyclonal antisera. Firstly, there are reports that there is no difference in the polyclonal response to chicken apo-RCP and vitamin bound-RCP (Cotner, 1972; Ramanathan *et al.*, 1979, 1980), despite the significant conformational changes that occur on binding of the vitamin at the active site. If tryptophan is critically involved in the binding of riboflavin to chicken RCP (Blankenhorn, 1978), and since it appears that 5 out of 6 of the tryptophan residues in chicken RCP are contained in hydrophobic pockets of the molecule (residues 54, 84, 106, 120, 156) (figure 3), these residues may not be exposed to the surface of the molecule and could thus account for the non-immunogenicity of the riboflavin binding site. Another observation made using polyclonal antisera, is that there is no difference in antigenicity of egg yolk RCP and egg white RCP (Cotner, 1972). Egg

Future prospects

We are currently characterising a number of other MAbs and attempting to delineate the regions of their interaction with the chicken RCP molecule. By treating the native protein with trypsin or cyanogen bromide (CNBr), a number of peptides are produced some of which are recognized by a few MAbs (Kuzhandhaivelu, N., Karande, A. A. and Adiga, P. R., unpublished results). Sequencing of these peptides should indicate the exact regions on the chicken RCP molecule which interact with the antibodies. Hopefully, a few of the peptides generated from the chicken RCP molecule could be used as immunogens to generate antibodies which might cross-react with the native protein. A particularly attractive candidate for this could be the phosphopeptide which has been shown to be involved in uptake of chicken RCP by the oocyte. It is attractive to speculate that antibodies to this peptide could inhibit the binding of the native protein to the putative placental receptor thereby resulting in reduced uptake of the vitamin by the fetoplacental unit in pregnant mammals.

Consequent to a complete understanding of the antigenic structure of chicken RCP, the MAbs could be used to probe further into the regions of homology in the mammalian proteins. The ability of MAbs to detect a single amino acid change in protein sequence should enable detection of the evolutionary conservation and divergence in the sequences of mammalian RCPs. Preliminary observations do indeed indicate that certain epitopes on mammalian RCPs are less conserved than others (Visweswariah, 1986). To confirm these observations, the cloning of chicken RCP cDNA is in progress. Cloning of the chicken RCP gene from a chicken liver cDNA library, followed by sequencing of the gene and hybridisation studies with rat and human genomic libraries should again substantiate the prediction regarding the extensive evolutionary conservation of the carrier protein. A few examples of proteins that have been conserved to a high degree are known. RCP joins this list because it is a protein whose physicochemical, immunological, functional and biosynthetic characteristics appear to remain grossly unchanged during the transition from oviparity to viviparity.

Acknowledgements

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Effect of luteinizing hormone releasing hormone analogues on testosterone metabolism *in vitro*—A study with mature rat ventral prostates

A. MAITRA, R. JOSEPH, A. R. SHETH[†], N. A. SHETH[*] and
J. JOSEPH[**]

Institute for Research in Reproduction, Jahangir Merwanji Street, Parel, Bombay 400 012, India

[*]Cancer Research Institute, Parel, Bombay 400 012, India

[**]Magyar Tudomanyos Akademia, Hungarian Academy of Sciences, 1361 Budapest, V_1 Roosevelt Ter 9, Budapest, Hungary

Abstract. The effects of two luteinizing hormone releasing hormone analogues (a superagonist and an antagonist) on the conversion of testosterone to dihydrotestosterone in homogenates prepared from adult rat ventral prostates were studied. At higher doses, the superagonist showed a significant dose-dependent inhibition of the conversion of testosterone to dihydrotestosterone. In comparison, the antagonist showed only a marginally inhibitory trend. The implications of these observed effects *vis-a-vis* the use of the analogues in the endocrine management of prostatic cancer have been discussed.

Keywords. Prostate; LHRH analogues; testosterone metabolism; 5 α-reductase activity.

Introduction

Since 1971, when Schally and associates elucidated the structure of naturally occurring luteinizing hormone releasing hormone (LHRH), numerous synthetic analogues have been developed, many of which have been found to have greatly increased potency compared to naturally occurring LHRH (Joseph and Smith, 1987). Chronic administration of pharmacologic doses of LHRH and its analogues has been demonstrated to inhibit steroidogenesis in a variety of species (Trachtenberg, 1982). LHRH compounds, in combination with pure antiandrogen flutamide have been used in bringing about a hypoandrogenic state in patients with advanced stages of prostate cancer. However, a clear understanding of the effect of these compounds on the prostate still needs to be determined in order to confirm its use as a pharmacologic agent of castration. In this context steroidogenic conversions in the prostate, mainly conversion of testosterone to dihydrotestosterone, would be of importance since DHT has been found to be a useful marker for antiandrogen therapy in prostate cancer (Geller *et al.*, 1984). No studies have so far been available on this aspect. A preliminary study carried out by us using a synthetic LHRH demonstrated some increase in the *in vitro* conversion of testosterone to DHT in immature rat ventral prostates. This change was however not significant (Sheth *et al.*, 1987b). It was interesting therefore to extend the study to LHRH analogues in order to gain insight into the interaction of these compounds with steroidogenic

Materials and methods

Seventy five day old male rats of Holtzman strain were used. The ventral prostates of these rats were excised under ether anaesthesia and processed as described by Sheth *et al.* (1987a). However, instead of using minced prostate tissue, the prostates were pooled and homogenized such that 1 ml of the homogenate contained 40 mg of the prostate tissue. Conversion of testosterone *in vitro* in the presence of NADPH was carried out using 1 ml of homogenate in each tube. The procedures involving *in vitro* conversion, extraction, separation and quantitation of 5α-reductase activity have been described in detail earlier (Joseph *et al.*, 1987; Sheth *et al.*, 1987a).

Using this method we determined the effect of Ovurelin^R a gonadotropin releasing hormone superagonist, and HB235, an LHRH inhibitor, on the 5α-reductase activity from mature rat prostates. As before, the results are described in terms of the amount of testosterone reduced to its major metabolite, DHT.

The validity criteria employed to validate the method have been fully described earlier (Sheth *et al.*, 1987a).

Results

Table 1 presents data indicating 5α-reductase activity obtained in the presence of 4 different doses of the superagonist Ovurelin and in the absence of this compound (control). At the lower doses used (25 and 50 ng) no significant changes were observed compared to the control either in the amount of testosterone reduced or in percentage conversion. However, at higher doses (75 and 250 ng), both parameters showed a clear declining trend. At 250 ng, the decrease was significant with respect to the control as well as the 25 and 50 ng doses. At 75 ng the amount of testosterone

Table 1. Effect of LHRH superagonist (Ovurelin) on testosterone metabolism in mature rat ventral prostate *in vitro*.

Ovurelin concentration (ng/10 mg tissue)	Testosterone reduced (p mol/10 mg tissue)	Percentage of testosterone reduced/10 mg tissue	DHT cpm/T cpm
0 (4)	286 ± 19.84	39.5 ± 1.32	0.14 ± 0.012
25 ng (3)	290 ± 19.51	42.67 ± 2.85	0.115 ± 0.02
50 ng (4)	272 ± 14.47	40.25 ± 2.01	0.094 ± 0.009^a
75 ng (4)	232 ± 12.46^b	34.0 ± 1.78^c	0.110 ± 0.008
250 ng (3)	219 ± 7.75^d	28.67 ± 1.20^e	0.073 ± 0.004^f

All values are mean $\pm$ SE of mean.

Figures in parentheses indicate the number of determinations.

[a] Significantly lower than control ($P < 0.05$).

[b] Significantly lower than 25 ng ($P < 0.05$).

[c] Significantly lower than control ($P < 0.05$); 25 ng ($P < 0.05$).

[d] Significantly lower than control ($P < 0.05$); 25 ng ($P < 0.05$); 50 ng ($P < 0.05$).

[e] Significantly lower than control ($P < 0.01$); 25 ng ($P < 0.05$); 50 ng ($P < 0.001$).

ng also the decrease was significant with respect to the control; at other doses
less significant.

LHRH antagonist HB235 did not cause any significant change with respect to
ontrol either in the amount of testosterone reduced or in the percentage
rsion (table 2). However, the DHT/testosterone ratios do indicate an inhibition
conversion of testosterone to DHT at higher doses (75 and 250 ng).

Table 2. Effect of LHRH antagonist (HB235) on testosterone metabolism in mature rat
ventral prostate *in vitro*.

HB235 concentration (ng/10 mg tissue)	Testosterone reduced (p mol/10 mg tissue)	Percentage of testosterone reduced/10 mg tissue	DHT cpm/T cpm
0 (7)	$141 \pm 12 \cdot 56$	$38 \cdot 71 \pm 3 \cdot 99$	$0 \cdot 147 \pm 0 \cdot 026$
25 (3)	$139 \pm 10 \cdot 40$	$39 \cdot 0 \pm 3 \cdot 51$	$0 \cdot 137 \pm 0 \cdot 008$
50 (7)	$158 \pm 5 \cdot 06$	$41 \cdot 57 \pm 2 \cdot 03$	$0 \cdot 179 \pm 0 \cdot 027$
75 (4)	$151 \pm 13 \cdot 66$	$40 \cdot 25 \pm 4 \cdot 13$	$0 \cdot 086 \pm 0 \cdot 017$[a]
250	$119 \pm 12 \cdot 75$[b]	$31 \cdot 67 \pm 4 \cdot 37$[c]	$0 \cdot 056 \pm 0 \cdot 001$[d]

All values are mean $\pm$ SE of mean.

Figures in parentheses indicate the number of determinations.

[a] Significantly lower than 25 ng ($P < 0 \cdot 05$); 50 ng ($P < 0 \cdot 05$).

[b,c] Significantly lower than 50 ng ($P < 0 \cdot 05$).

[d] Significantly lower than control ($P < 0 \cdot 05$); 25 ng ($P < 0 \cdot 001$); 50 ng ($P < 0 \cdot 001$).

s a comparison of the two analogues tested shows that the superagonist has a
cant dose-dependent inhibitory effect on the 5α-reductase activity at higher
the effect of the antagonist is relatively marginal.

ssion

ethodology adopted in the present study is based on the major assumption
rat prostate tissue more than 90% of testosterone is converted to DHT. This
en previously demonstrated under different experimental conditions by Massa
artini (1974) and later confirmed by Purvis *et al.* (1986). The results obtained
previous study with LHRH using immature rat prostates did not show any
cant changes although a trend towards augmentation of the 5α-reductase
y was perceptible. In this context, it is interesting to observe that both the
analogues tested in the present study showed an inhibitory effect on the *in*
onversion of testosterone to DHT in mature rat prostate tissue homogenate,
gh the extent of inhibition varied greatly. The agonist showed a predominantly
r inhibition of the 5α-reductase activity than the antagonist under similar
ions.

1979). As stated by these authors, the potential usefulness of any antiandrogenic agent would be determined by the DHT levels in prostate tissue. The level of DHT would depend upon the following factors—plasma testosterone substrate, 5α-reductase, 3-oxidoreductase and receptor binding, all essential biochemical steps for the mediation of androgen action. Therefore inhibition of the 5α-reductase activity observed in our study is interesting, since it would lead to a decline in the tissue DHT store. However, to obtain any conclusive evidence, the other factors mentioned above also need to be investigated. Studies on the effect of LHRH analogues on steroidogenic enzymes in the testis have been extensively carried out but such studies (for the prostate) are few. Using the agonistic analogue Buserelin, Trachtenberg (1982) demonstrated that in spite of 90% reduction in serum androgen concentration, prostatic weight and prostatic androgen receptor content remained largely unchanged. Detailed studies on similar lines need to be carried out using Ovurelin and HB 235 in order to determine their potential in prostate cancer therapy.

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Keratinization of rat vaginal epithelium. II. Immunofluorescence study on keratin filaments in cycling and estrogen primed rats

S. VIJAYASARADHI* and P. D. GUPTA†

Centre for Cellular and Molecular Biology, Hyderabad 500 007, India
*Present address: Memorial Sloan-Kettering Cancer Centre, 1275 York Avenue, New York 10021, USA

Abstract. Rat vaginal epithelial layers from animals in different phases of the estrous cycle showed positive immunofluorescence when treated with either monoclonal antibody to intermediate filaments or immunoglobulin G fraction of antiserum raised against epidermal keratin filaments. During estrus, the intensity of fluorescence observed was maximum in the keratinized cellular layers. In estradiol-primed immature and ovariectomized rats the maximum fluorescence intensity was observed in the layers immediately lining the lumen. However, basal layers in ovariectomized rats also showed some fluorescence. Data presented in this communication indicate that the abundance of keratin filaments in vaginal epithelial cells can be modulated by altering the level of estradiol in the system.

Keywords. Immunofluorescence; electron microscopy; keratin filaments; intermediate filaments; estradiol; vaginal epithelium.

Introduction

A family of intermediate filaments (IF) from various species has been characterized on the basis of biochemical and immunological properties (Aynardi et al., 1984). One type of cell may contain more than one class of intermediate filaments (IF) (Osborn et al., 1980; Henderson and Weber, 1981). One of the types of IF resembles the tonofilaments seen in epithelial cells (Brysk et al., 1977). Most of the recent work on keratin filaments in vivo and in vitro has been done mainly on skin and uterine epithelial cells (Aynardi et al., 1984; Eichner et al., 1985). Keratinization of vaginal epithelial cells (VEC) depends on the level of circulating estradiol in the system (Kronenberg and Clark, 1985a, b; Vijayasaradhi and Gupta, 1987). Recently we have demonstrated that rat VEC in vitro also keratinize and show characteristic microridges in the presence of estradiol in the culture medium (Vijayasaradhi et al., 1987). We have also shown that in vivo the rats become responsive to estradiol as early as 10 days after birth; however, microridges on the surface of VEC are seen only from day 60 (Vijayasaradhi and Gupta, 1987).

The present study was based on the use of specific antibodies to keratin filaments and was initiated to obtain more information on (a) responsiveness of cell type to estradiol, (b) distribution of keratin filaments in various layers of the vaginal epithelium in different stages of the estrous cycle and in immature and adult ovariectomized rats primed with estradiol and (c) cross-reactivity between keratin and IF. The intracellular distribution of keratin filaments (tonofilaments) in vaginal epithelial cells (VEC) was also studied using electron microscopic techniques.

†To whom all correspondence should be addressed.

The estrous cycle of adult Wistar rats was assessed daily by vaginal smears taken between 1000 and 1200 h. Only those animals exhibiting two consecutive normal estrous cycles were used for the present study (Vijayasaradhi and Gupta, 1987). Bilateral ovariectomy was performed on the randomly chosen adult cycling rats. Vaginal smears of ovariectomized rats were examined for at least two weeks after the operation. Immature (30 day old) and ovariectomized rats were primed with estradiol-17β (0.1 μg/g body weight) obtained from Sigma Chemical Co., St. Louis, Missori, USA. Vaginae were excised quickly from adult cycling, immature and ovariectomized estrogen-primed and control rats. Small pieces of the tissue were placed vertically, sandwiched between two rectangular blocks of liver dissected out from the same animal. The sandwiches were mounted on tissue holders and immediately placed on the pre-cooled metallic block ($-20°C$) in the chamber of an American Optical Histostat microtome. After leaving the tissue in the chamber for about 1 h, 5–10 μm thick sections were cut. These sections were collected on clean glass coverslips and stored at $-20°C$ until further use. Before staining, the tissue sections were allowed to reach room temperature. The sections were incubated for localization of keratin filaments using the sandwich technique (Gupta, 1983). Briefly, the sections were incubated in either the monoclonal antibody to IF (culture supernatant at 1:10 dilution) or the immunoglobulin G (IgG) fraction of rabbit antiserum to epidermal keratin (dilutions 1:50 to 1:100). The 1:100 dilution gave good results. One mg/ml bovine serum albumin was added to the antiserum. The sections were incubated in diluted antibody for 30 min at room temperature ($30° \pm 2°C$) in a humid chamber. After incubation the sections were washed with phosphate buffered saline (PBS) and reincubated in fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG at 1:10 dilution or goat anti-rabbit IgG-FITC at 1:10 dilution. After incubation for 15–20 min at room temperature, the sections were washed thoroughly with PBS, dried and mounted in 70% glycerol in PBS (pH 8).

To check the specificity of the fluorescence reaction the following control experiments were performed:

(a) *Antibody control*: (i) Normal rabbit serum was used instead of antiserum against keratin filaments. (ii) The treatment with first antibody (antibody to IF or keratin filaments) was avoided, in other words, the sections were directly incubated in rabbit anti-mouse IgG-FITC or goat anti-rabbit IgG-FITC as described above. In these sets of experiments no fluorescence was detectable.

(b) *Hormone control*: Immature and ovariectomized rats not treated with estradiol were used. In these sets of experiments some diffuse fluorescence was seen.

The sections were examined in a Polyvar (Reichert-Jung) microscope with a 425–475 nm excitation filter and at 510 nm barrier filter. Ilford FP4 (125 ASA/22 DIN) film was used for photography.

Small pieces of vaginae from normal adult cycling, immature control and estradiol-primed, and adult ovariectomized control and primed rats were fixed in 3% gluta-raldehyde, post-fixed in 1% osmium tetroxide, washed, dehydrated and embedded in Araldite (Gupta, 1983). Ultrathin sections were examined in a Jeol 100 CX electron

sections of vaginal epithelium obtained from proestrus, estrus, diestrus, primed and control rats were stained by the indirect immunofluorescence for locating keratin (intermediate) filaments. Essentially, there is no signi-fference between the data obtained using monoclonal antibody to IF and sing the IgG fraction of rabbit antiserum to epidermal keratin. The nal antibody stained the filaments in tissue from all phases of the estrous owever, distinct differences in the distribution of these filaments were clearly . In proestrus tissue, all the cell layers exhibited a staining pattern of uniform evenly distributed in the cytoplasm (figure 1). The basement membrane n intense fluorescence. There was no reaction in the nuclei. In estrus tissue al layers did not show any cellular details since they were keratinized at this n intense fluorescence was localized in the cell layers projecting towards the less intense fluorescence in the cells of basal and intermediate zones was erved (figure 2). However, here also basement membrane shows an intense fluorescence. Diestrus epithelium closely resembled the proestrus epithelium ining pattern (figure 3). However, the intensity of the fluorescence was signi-ower in diestrus compared to that in proestrus and estrus. Again, in diestrus lar details were clear even in the superficial layers. Diffuse fluorescence was in the cytoplasm of the cells; the nuclei remained free from it.

s on the intracellular distribution of tonofilaments in various phases of the cycle and in immature control and estradiol-primed as well as in adult omized and estradiol-primed VEC showed that tonofilament bundles are only in the cytoplasm. During early developmental stages no tonofilament are seen. However, after estradiol treatment tonofilament bundles appear in . Similarly ovariectomized rat VEC do not show these bundles but primed omized rat VEC do show these bundles in the cytoplasm (figures 4–6).

liol-primed immature rats show distinct differences in the distribution of fluorescence from that of estradiol-primed adult ovariectomized animals. ue from the vehicle injected 30-day-old control animals, a diffuse fluorescence ent in all the 3 cell layers (superficial, intermediate and basal) of the vaginal m (figure 7). Twelve h after estradiol administration, intense fluores-as observed towards the stromal side of the basal epithelial cells. A few the intermediate layer were also stained intensely (figure 8). Basement ne also stained strongly positive. In tissue from the ovariectomized vehicle control, the basal cells were stained brightly (figure 9); administration of the resulted in an increase in the intensity of fluorescence in the superficial iating cell layers. However, stromal cell layers also showed positive nce (figure 10).

on

ready been shown by several workers that epidermal pre-keratin and keratin belong to the IF family (Eichner et al., 1985; Sun et al., 1985). Epidermal filaments differ from the vaginal keratin filaments (VKF) at least in one
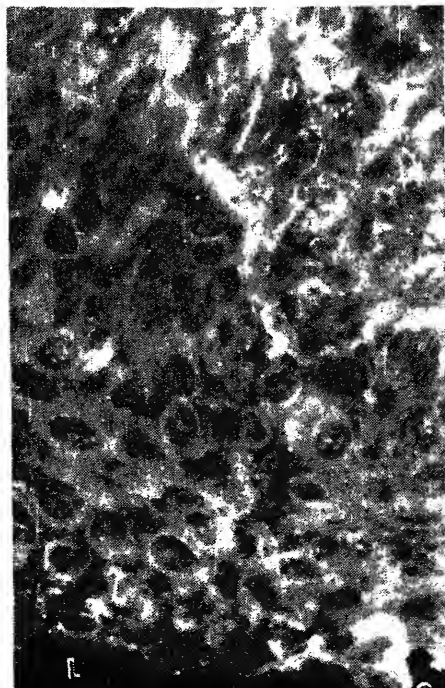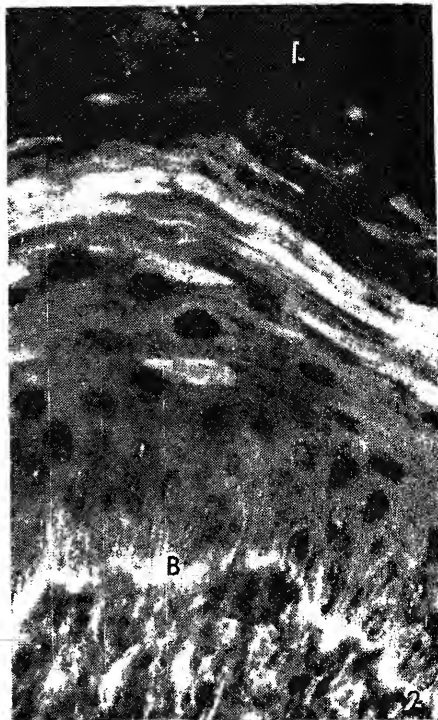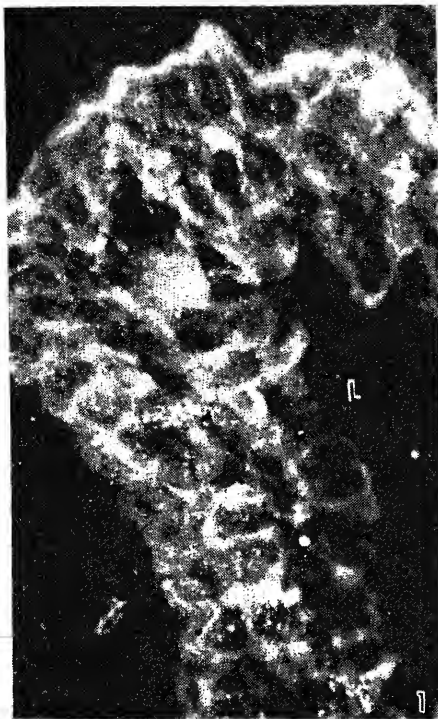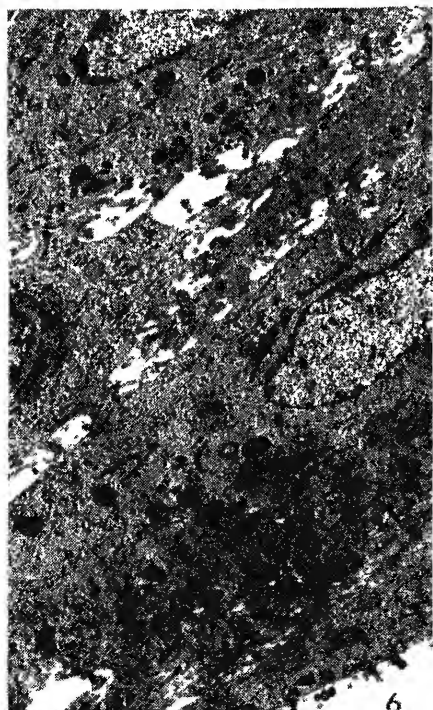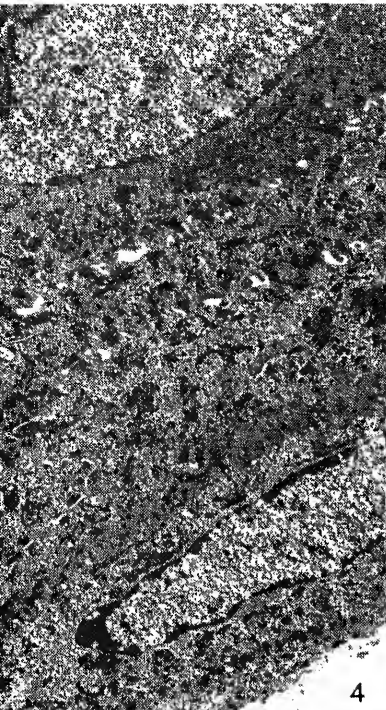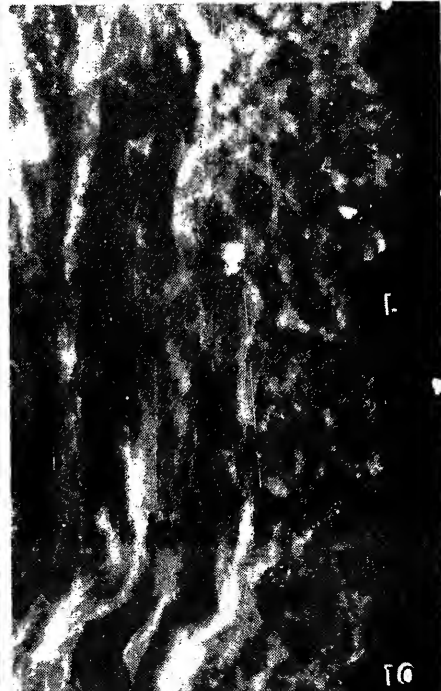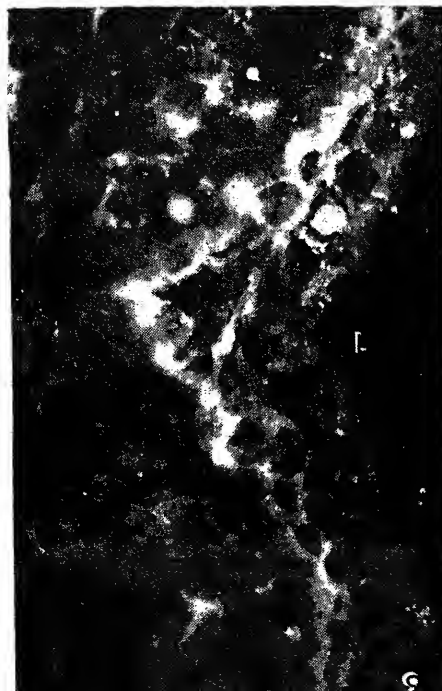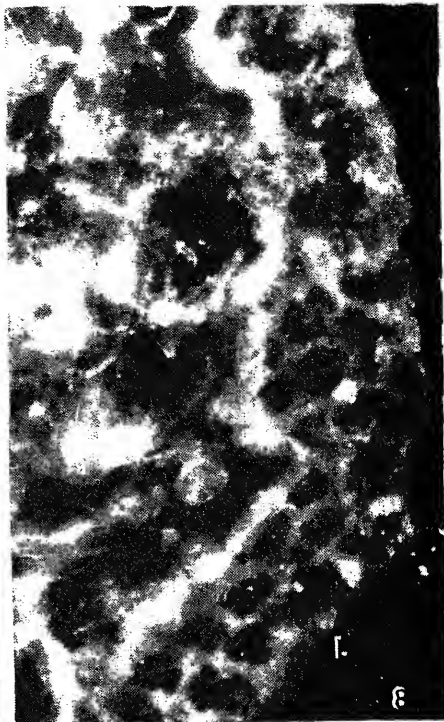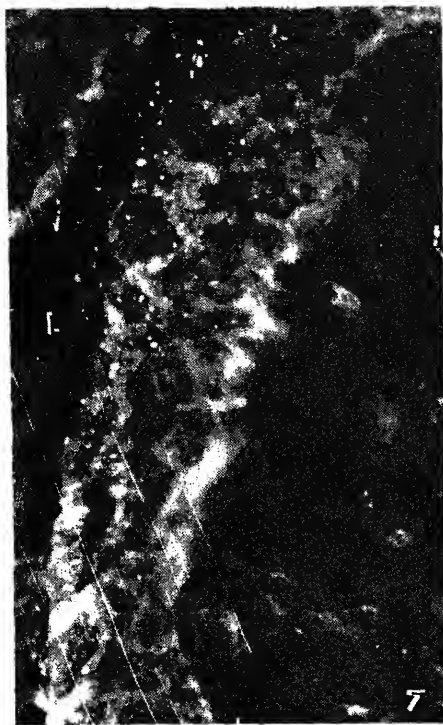
heless, antibodies to IF or epidermal keratins cross-react with vaginal keratin
ts. Using these antibody probes we have shown that vaginal keratin also
s to the IF family, at least on the basis of immunological cross-reactivity. Our
lished data based on sodium dodecyl sulphate-polyacrylamide gel electro-
ic analysis of vaginal epithelial cell extracts from immature rats indicate the
ce of keratin polypeptides of lower molecular weight (about 46–55 kDa)
s adult estrus rats and immature rats primed with estradiol show higher
lar weight polypeptides (about 52–68 kDa).

ntly, Kronenberg and Clark (1985a, b) and our group (Gupta *et al.*, 1986;
saradhi *et al.*, 1987; Vijayasaradhi and Gupta, 1987) have shown using *in vivo*
vitro models that the keratinization of rat VEC depends on the level of
ol in the system. The present immunofluorescence study, after administration
diol to immature (30 day) and ovariectomized adult animals also reveals that
mary response to the hormone leads to proliferation of cells and enhanced kera-
thesis in the cells of the basal layer. In the ovariectomized adult animals the
y response appears to be differentiation of intermediate layers with increased
synthesis. These observations indicate a possible difference in the
station of primary response to estradiol between the vaginal epithelium of
s exposed for the first time and those which were exposed before, but were
d of the hormone by ovariectomy. Further, during the estrous cycle, the
um intensity of the fluorescence is seen in estrus when the circulating estradiol

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Figures 1–3. Frozen tissue sections incubated in monoclonal antibody to intermediate
filaments. Rabbit antimouse IgG-FITC was used for fluorescent staining. **1.** Vagina from
proestrus rat. Basement membrane shows an intense positive reaction. Cell cytoplasm of
basal and intermediate layers react positively (× 1700). **2.** Vagina from estrus rat. Upper
cornified layers show intense positive fluorescence. Intermediate layers do not show much
reaction while basal layers show moderately positive reaction (× 1200). **3.** Vagina from
diestrus rat. All cells show a diffuse positive fluorescence except the basement membrane
(× 1400).
L, Lumen; B, basement membrane.

Figures 4–6. Transmission electron micrographs of thin sections of vaginal epithelial cells.
Mainly upper (luminal) layers are shown. **4.** Vagina from estrus rat. Tonofilaments (in
form of bundles) are distributed in the cytoplasm (× 9000). **5.** Vagina from 30 day old
hormone-primed rat. Though no filaments are seen in the cytoplasm, desmosomes and
membrane complex are seen. Vaginal tissue from control rats does not show any such
features (× 30,000). **6.** Vagina from diestrus rat. Tonofilaments are not so conspicuous
(× 8,000).
T, Tonofilaments; N, nucleus; L, lumen.

Figures 7–10. Frozen tissue sections incubated with monoclonal antibody to intermediate
filaments. Rabbit antimouse IgG-FITC was used for fluorescent staining. **7.** 30 day old rat
vagina. Diffuse fluorescence is seen in all cells. Basement membrane show an intense positive
fluorescence (× 1800). **8.** 30 day old rat vagina, primed with estradiol-17β. Besides VEC,
basement membrane and stromal cells also show an intense fluorescence (× 2000).

higher fluorescence intensity compared to diestrus cells. This again reflects the level of circulating estradiol in the two phases of the cycle.

The functions of IF are poorly understood (Goldman *et al.*, 1985; Weber and Giesler, 1985). In stratified squamous epithelia, the keratin or tonofilament sub-class of IF accounts for 30% or more of cell protein. These filaments form a dense, insoluble intracellular matrix during terminal differentiation that aids in the protective barrier function of the epithelia. However, no function has been assigned with certainty to the keratin filaments that are abundant in many other epithelia (Franke *et al.*, 1978, 1981; Sun *et al.*, 1979).

There is good agreement between electron microscope and fluorescence microscope studies on the intracellular distribution of tonofilament (electron microscopy) and IF (immunofluorescence) in the vaginal epithelium. The fluorescence intensity is directly proportional to the abundance of tonofilament in the cytoplasm of the VEC. The cells showing diffuse fluorescence also do not show many tonofilament bundles.

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cogen metabolism in human fetal testes

C. DATTA, K. MISRA, J. DASGUPTA, T. SENGUPTA, S. DE and D. SENGUPTA*

Department of Biochemistry, University College of Science, University of Calcutta, 35, Ballygunge Circular Road, Calcutta 700 019, India

Abstract. The ontogeny of glycogen synthetase, glycogen phosphorylase and α-D-glucosidase, enzymes which are associated with glycogen metabolism and glycogen level has been studied in human fetal testes of gestational age ranging from 14–32 weeks. Glycogen synthetase activity reaches the peak value at 17–20 weeks of gestation, thereafter it decreases. α-D-Glucosidase activity increases with the advancement of pregnancy up to 28 weeks of gestation decreasing thereafter very rapidly. Phosphorylase activity remains more or less constant throughout gestation. The maximum increase in glycogen content at early stages of gestation (17–20 weeks) and gradual reduction with the advancement of pregnancy are correlated with histochemical observation by the periodic acid-Schiff technique.

Keywords. Glycogen; glycogen synthetase; glycogen phosphorylase; α-D-glucosidase; fetus; fetal Leydig cell.

duction

ogen is the main source of energy in the animal reproductive system. The largest ion of testicular glycogen is contained in the seminiferous tubule. However, its ibution is not uniform and varies according to the degree of maturity of niferous epithelium (Gierke, 1937). It plays an important role in the maturation rm cells. Intra-tubular glycogen is abundant in the prepubertal stage, diminishes ingly with the beginning of puberty, and reappears during the period of sexual iration (Fabbrini *et al.*, 1969). The glycogen level in the seminiferous tubule /s a cyclic behaviour during spermatogenesis at puberty acting as a source of gy in the synthesis of DNA (Re, 1974). The activity of active phosphorylase, low epubertal life (Re *et al.*, 1973), rises during the pubertal phase (Seilicovich and et, 1973) and is very high during spermatogenesis (Mangan and Mainwaring,). In prepubertal testes, in the absence of DNA synthesis, glycogen is not used use of the lack of activation of phosphorylase (Re, 1974).

espite all the evidence regarding the existence and function of glycogen in loping mammalian testes little attention has been paid to glycogen metabolism uman fetal testes. The present study was therefore undertaken to measure the ities of glycogen synthetase, α-D-glucosidase and phosphorylase and the levels ycogen (histochemically and biochemically) in human fetal testes throughout the ation period.

All chemicals and reagents used in this study were of analytical grade (E. Merck, Germany and British Drug House, UK). Fine chemicals were purchased from Sigma Chemical Co., St. Louis, Missouri, USA.

Collection of samples

The fetuses were obtained from therapeutic abortions (up to 20 weeks from conception) from different nursing homes and MTP (medical termination of pregnancy) clinics in and around Calcutta. Fetuses above 20 weeks were obtained from stillbirths. The ages of the fetuses were calculated from the mother's menstrual cycle histories and from crown-rump and crown-heel lengths of the fetuses. The method provides data correct to within a week in the majority of cases (Iyengar, 1973). According to gestational age the fetuses were grouped as follows: group I, 14–16 weeks; group II, 17–20 weeks; group III, 21–24 weeks; group IV, 25–28 weeks and group V, 29–32 weeks. Fetuses which showed intrauterine growth retardation were excluded. Both the testes were removed immediately after collection and stored in a freezer ($-20°C$) for later use.

Human adult testes were obtained from NRS Hospital, Calcutta about 2–4 h after death from (according to available information) healthy persons (25–35 years old) who died in accidents other than testicular injury. The post-mortem stability of the enzymes was frequently checked over a period of 1–8 h and no appreciable changes could be detected.

Enzyme assays

Glycogen synthetase was assayed by the method of Rogers *et al.* (1963). The method of Jauhiainen and Vanha Perttula (1985) was followed for the determination of α-D-glucosidase activity using *p*-nitrophenyl-α-D-glucopyranoside as the substrate. Phosphorylase activity was measured by the method of Freedland *et al.* (1968) in the presence of 0·02 mM adenosine monophosphate (AMP). For the estimation of active phosphorylase AMP was excluded from the assay system. Glycogen was determined according to the method of Seifter *et al.* (1950). Protein was determined by the method of Lowry *et al.* (1951). The homogenate was kept at $0°–4°C$ throughout.

For histochemical studies the fetuses were divided into the following groups: early gestation group, 16–20 weeks; mid-gestation group, 21–24 weeks and late gestation group, 25–28 weeks. Both the testes were dissected out as quickly as possible and one of them was fixed in Carnoy's fluid. The presence of glycogen was confirmed by periodic acid Schiff (PAS) staining (Pearse, 1968).

Results

The results presented in table 1 indicate that glycogen synthetase in human fetal testes increases gradually, peaking at 17–20 weeks of gestation and then decreases as

onal age	Glycogen (μg/mg tissue)	Glycogen phosphorylase (nmol of P_i liberated/min/mg protein)	Glycogen synthetase (nmol of UDP liberated/min/mg protein)	α-D-Glucosidase (nmol of product liberated/min/mg protein)
6)	0·34 ± 0·02	0·98 ± 0·06	1·02 ± 0·09	1·30 ± 0·07
5)	0·57 ± 0·04*	1·12 ± 0·07	1·63 ± 0·11*	1·34 ± 0·07
5)	0·53 ± 0·04	1·24 ± 0·09	0·49 ± 0·03*	1·56 ± 0·12
5)	0·18 ± 0·009*	1·33 ± 0·07	0·48 ± 0·03	1·62 ± 0·10
4)	0·11 ± 0·005*	1·42 ± 0·08	0·45 ± 0·04	0·67 ± 0·03*
3)	0·75 ± 0·05	3·03 ± 0·28	1·26 ± 0·01	0·13 ± 0·01

les are mean ± SE of mean.
rs in parentheses indicate the number of cases studied.
cant difference (from preceding value), $P < 0.001$ (Student's t test).

eks); the activity then increases and reaches its maximum level at 25–28 weeks
tation. However specific activity falls thereafter until the adult level is reached.
otal phosphorylase activity does not alter significantly throughout gestation in
n fetal testes and no significant amount of active phosphorylase could be
ed throughout the gestational period. In the adult testes active phosphorylase
y was detected.

tochemical examination of human fetal testes (16 weeks gestation) (figure 1)
s that PAS reaction is little in seminiferous cords and interstitial cells but
albuginea reacts more strongly. In mid gestation (21–24 weeks) (figure 2), PAS
on was found to be less compared to that in the early weeks of gestation. At
stages of gestation (25–28 weeks) (figure 3) PAS reaction was observed to be
aint in seminiferous cords and interstitial cells as well as in tunica albuginea.
observations are in agreement with the biochemical observation that the
en content of developing human testes is high at 17–20 weeks of gestation and
ally decreases with the advancement of pregnancy.

ssion

ell known that human primordial germ cells contain a large cytoplasmic store
cogen. In 17–30 day-old human embryos glycogen content remains high in the
rdial germ cells (Witschi, 1948). In 44–48-day embryos the primordial germ
ave less glycogen and glycogen also incorporated into the testicular cords.
the formation of tunica albuginea, glycogen deposits are observed among the
lial cells in both portions of the testicular cords. Fetal Leydig cells also contain
positive glycoprotein granules. Gillman (1948) stated that Leydig cells are
lant in fetal life, decrease in postnatal life and increase again at puberty,
mably differentiating from undifferentiated interstitial cell (Hayashi and
son, 1971). Shortly after birth the glycogen content of primordial germ cells
ishes (Falin, 1969; Fujimoto et al., 1977). The results presented here (table 1,
s 1–3) reveal that the glycogen content of human fetal testes decreases with

(1)

(2)
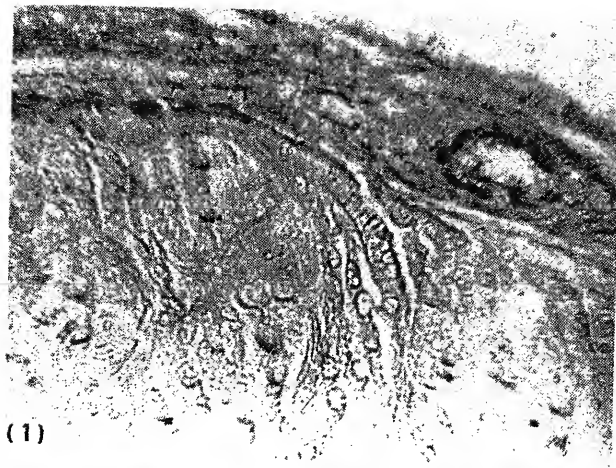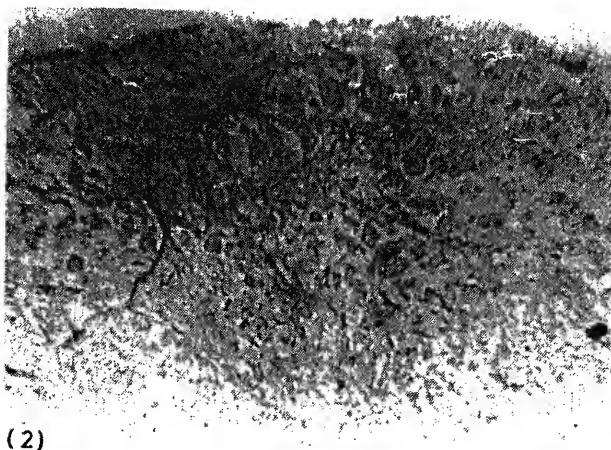
n primordial germ cells with progress in pregnancy may reflect its utilization to
metabolic requirement (Bellve, 1979). The results presented here (table 1) also
ate that the maximum glycogen synthetase activity is also during the period
the glycogen level is maximum *i.e.*, at 17–20 weeks of gestation. Not only was
phosphorylase activity found to be very low, but active phosphorylase was also
etectable throughout pregnancy (results not shown in the table). Contrary to
ctive phosphorylase activity is very low in prepubertal life (Re *et al.*, 1973) rises
g the pubertal phase (Seilicovich and Lloret, 1973) and is very high during
natogenesis in adult testes indicating the utilisation of glycogen by phosphory-
Table 1 also indicates that adult testes have a high activity of active
ohorylase and this result supports the earlier observations.

D-Glucosidase (also known as maltase) catalyzes the cleavage of α-D-glucose
ies from poly- and oligosaccharides. This enzyme has some role in glycogen
oolism (Rao *et al.*, 1971). Jauhiainen and Vanha Perttula (1985) have suggested
α-D-glucosidase may function in the digestion of absorbed polysaccharides and
protein as well as in the processing of glycoprotein synthesized by the testis.
high activity of α-D-glucosidase in human fetal testes at 25–28 weeks of
tion suggests that this enzyme is associated with the utilisation of glycogen
e in adult tissue where glycogen is utilised by phosphorylase *a*.

conclusion, it can be stated that glycogen, an important metabolic fuel store, is
1ally built up at early stages of development in human fetal testes and its level
s according to the energy needs at different stages of development. α-D-
osidase, along with phosphorylase, is involved in glycogen utilisation of
during fetal life.

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action of lanthanum chloride with human erythrocyte membrane in
on to acetylcholinesterase activity

SUNIL MUKHOPADHYAY, SILA MUKHOPADHYAY,
DILIP K. BHATTACHARYYA* and GORA CHAND CHATTERJEE[†]
Department of Biochemistry, University of Calcutta, 35, Ballygunge Circular Road,
Calcutta 700 019, India
*The Society for Research on Haematology and Blood Transfusion, Corporation Place,
Calcutta 700 087, India

Abstract. Lanthanum chloride (1 mM) inhibits the activity of acetylcholinesterase *in vitro* in the human erythrocyte membrane. Lineweaver-Burk analysis indicates that lanthanum chloride induced inhibition of acetylcholinesterase activity is competitive in nature. The Arrhenius plot shows that the transition temperature of erythrocyte membrane-bound acetylcholinesterase is significantly reduced in the presence of lanthanum chloride. These results suggest that lanthanum chloride increases the fluidity of the erythrocyte membrane and this may be a cause of inhibition of membrane-bound acetylcholinesterase activity.

Keywords. Human erythrocyte membrane; lanthanum; acetylcholinesterase; Arrhenius plot.

uction

anum, a member of the light lanthanides, exists in the ionic form at low con-
tion. It binds to the phospholipid component of the erythrocyte membrane
cts at the outer periphery of the membrane, without penetrating it (Venugopal
uckey, 1978). It is known that changes in the membrane microenvironment
he activities of various membrane-bound enzymes (Beauregard and Roufogalis,
Nemat-Gorgani and Meisami, 1979; Gordon *et al.*, 1980) and it seems likely
anthanum-erythrocyte membrane interactions may lead to changes in the
ies of membrane-bound enzymes. Weiner and Lee (1972) have shown that
num inhibits the activity of erythrocyte membrane-bound Ca-activated
se. Another light lanthanide, holmium, inhibits the erythrocyte membrane-
l (Ca + Mg)-ATPase (Schatzmann and Tschabold, 1971). In the present
igation the possible changes in lipid-protein interactions of the human eryth-
membrane bound acetylcholinesterase (AChE) as a result of treatment with
num have been discussed in the light of Arrhenius parameters.

ials and methods

hemicals used in this study were commercially available analytical grade
ial. Acetylthiocholine iodide, 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) and

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om all correspondence should be addressed.

Indian Rare Earths Ltd., Rare Earth Division, Udyogmandal, India.

Membrane preparation and incubation

Whole blood was collected by venepuncture in acid citrate dextrose (ACD) from male healthy donors between 25 and 40 years of age. The whole blood was centrifuged at $600\,g$ for 10 min at 4°C and the plasma and buffy coat were removed by aspiration. Packed erythrocytes were suspended in 0·9% saline. The erythrocyte count was taken by light microscopy and the concentration was adjusted to 1×10^6 erythrocytes/ml. The erythrocyte suspension was incubated with 1 mM $LaCl_3$ solution at 37°C for 1 h. A control incubation was carried out with an equal volume of 0·9% saline instead of 1 mM $LaCl_3$. Incubation was stopped by the addition of ice-cold 0·9% saline and the erythrocytes washed thrice with cold saline to remove $LaCl_3$. Erythrocyte membranes were prepared from control and $LaCl_3$-treated erythrocytes according to the method of Kunimoto and Miura (1985).

Enzyme assay

AChE (EC 3.1.1.7) activity was measured spectrophotometrically in control and $LaCl_3$-treated erythrocyte membranes according to the method of Ellman et al. (1961). The final assay medium (3 ml) consisted of 0·29 mM DTNB, 0·5 mM acetylthiocholine iodide and 0·05 ml of the enzyme preparation in phosphate buffer. The rate of change of colour was measured at 412 nm. Assays were performed at temperatures varying from 10°–40°C with 2–5 degree intervals. For kinetic studies substrate concentrations were varied from 0·2–0·8 mM. The protein content of the enzyme preparations was estimated according to Lowry et al. (1951) using bovine serum albumin as standard.

Arrhenius plots

To obtain the Arrhenius plots, square root analysis of the data was first carried out and then the logarithms of the corrected specific activity values at each temperature were plotted against the reciprocal of absolute temperature. The value of transition temperature (TT) was read directly from the plot.

The Arrhenius equation was utilised to estimate the activation energics of the enzyme above and below the transition temperature.

The statistical significance of difference between the mean values of test and control reactions was determined by Student's t test.

Results

From the Lineweaver-Burk plot (figure 1) of erythrocyte membrane AChE activity in the presence of 1 mM $LaCl_3$ it appears that the lanthanide increases K_m by 1·9-fold
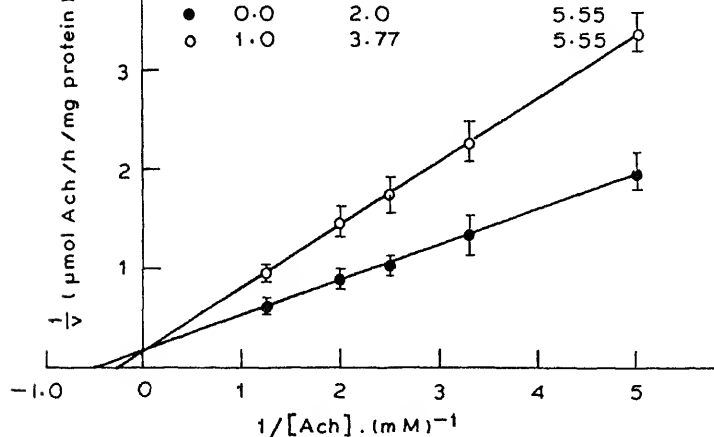
Figure 1. Lineweaver-Burk plots of AChE activity of human erythrocyte membrane in the absence and presence of LaCl₃. Each point represents mean of 5 independent experiments; bars are SD.

e activity of erythrocyte membrane-bound AChE was measured at various eratures (10°–40°C). It was found that the activity increased linearly with rise in erature in both LaCl₃-treated erythrocyte membrane and untreated erythrocyte rane (figure 2). However, the activity of AChE in LaCl₃-treated erythrocyte rane was consistently lower than the activity in untreated erythrocyte membrane the temperatures at which the enzyme activities were measured. When the results transformed into Arrhenius plots discontinuities in the slope became apparent in se of both LaCl₃-treated membrane and untreated membrane. Figure 3 shows rrhenius plot of AChE activity. Examination of the plots shows that the TT of erythrocyte membrane-bound AChE is 21·3°C and this is lowered to 18·5°C eatment of membranes with 1 mM LaCl₃ *in vitro*. Table 1 gives the apparent tion energies above and below the TT.

ssion

E is thought to be a peripheral extrinsic (Gordon *et al.*, 1980) phospholipo- n (Beauregard and Roufogalis, 1977) and the role of lipid, especially phos- pid, is vital for enzyme activity (Beauregard and Roufogalis, 1977). AChE the link between lecithin and protein in the erythrocyte membrane and ibutes to the maintenance of membrane integrity (Kutty *et al.*, 1976). These ers extended the fluid mosaic concept of membranes by proposing lipoprotein- in interaction and active participation of AChE in the maintenance of rane stability and function. Further it was proposed that the polar head s of lecithin form ionic bonds with the esteratic and anionic sites of the enzyme he free protein or the non-active site of the enzyme forms a protein-protein with structural proteins. The phospholipid component of biomembranes is
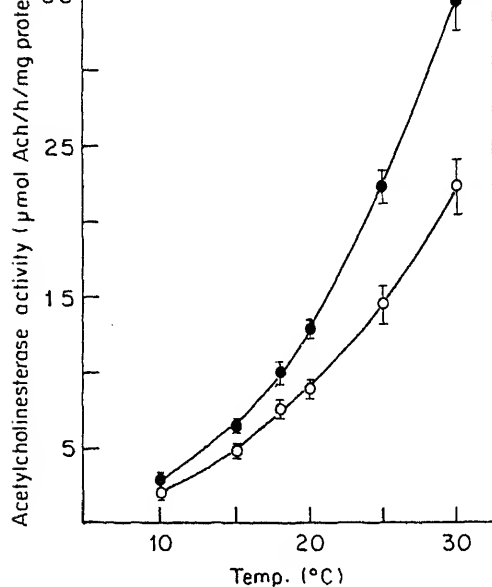
Figure 2. Effect of temperature on human erythrocyte membrane-bound AChE activity. (●), Control; (○), 1 mM LaCl₃.
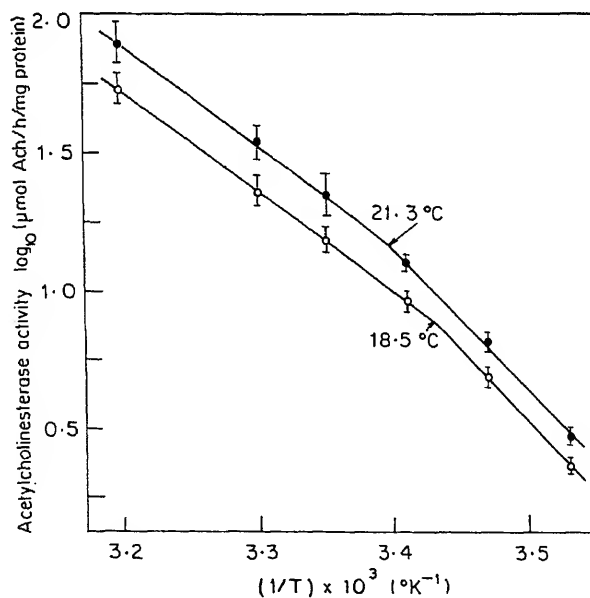
Figure 3. Arrhenius plots of human erythrocyte membrane-bound AChE activity. (●), Control; (○), 1 mM LaCl₃.

believed to be the site of action of La theride (Vannucci and Ludueña, 1978). H

LaCl$_3$ concentration (mM)	Transition temperature (°C)	Activation energy[a] (Kcal/mol)	
		Below TT	Above TT
0·0	21·3 ± 1·31	24·18 ± 1·20	15·55 ± 0·81
1·0	18·5 ± 1·10[b]	27·80 ± 1·22[b]	16·41 ± 0·92

Each result is the mean ± SD of 5 independent experiments.

[a]Calculated from the slopes of the lines in figure 3.

[b]Mean value significantly different from that of control, $P < 0.01$.

...ranes (Glick, 1976) as well as their structural organization (Elferink, 1977) may ...ponsible for the membrane specific effect of lanthanum on AChE activity.

...results of Lineweaver-Burk analysis suggest that lanthanum competitively ...s AChE activity in the erythrocyte membrane (figure 1). Decrease of substrate ... (K_m^{-1}) in the presence of lanthanum without any change in the catalytic ...ty (V_{max}) of erythrocyte membrane-bound AChE suggests that lanthanum ...at or close to the substrate binding site of the enzyme in such a way as to ...t the conformational change that normally occurs during catalysis (Miller and ..., 1975). AChE, like many other membrane-bound enzymes undergoes a ...tic change in apparent activation energy between 10° and 40°C. Although a ...r of explanations have been presented for this phenomenon, lipid-protein ...ctions have been often suggested as playing a major role (Ray et al., 1987). A ...n change in activation energy of membrane-bound enzymes at a particular ...rature, the TT, is generally indicated by a discontinuity in the Arrhenius plot. ...ystalline-to-liquid-crystalline phase transition of membrane lipids takes place ...temperature (Overath and Trauble, 1973; Grisham and Barnett, 1973). The ...erythrocyte membrane-bound AChE was found to be 21·3°C (figure 3). The ...num-induced decrease in TT indicates that the lipid phase transition of ...ocyte membrane takes place at a lower temperature in the presence of ...num. It is known that the thermal transition around 20°C in mammalian ...rane-bound enzymes indicates a change in the lipid fluidity of the membrane ...ka and Teruya, 1973; Kimelberg and Papahadjopoulos, 1974). A rise in TT is ...ted to be due to condensation, i.e. decrease in fluidity of the phospholipid ...or bilayer (Gordon et al., 1980). Thus the lowering of TT (table 1) of human ...ocyte membrane-bound AChE in the presence of lanthanum suggests that ...num increases the lipid fluidity of the membrane and thereby produces an ...ion of AChE activity.

...wledgements

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Antiserum directed against cell surface antigens is lethal to *Leishmania donovani* promastigotes

ARPITA DAS, SAHANA MAZUMDER and
SWADESH DUTTAGUPTA*

Indian Institute of Chemical Biology, 4, Raja S. C. Mullick Road, Calcutta 700 032, India

Abstract. The purified flagellar fraction of *Leishmania donovani* promastigotes consists of 30–35 polypeptides. Antiserum raised against this fraction reacts with both flagella and pellicular membrane antigens as evident from immunoblot and immunofluorescence studies. Only 3 of these immunoreactive polypeptides are flagellum-specific. The antiserum agglutinates the cells and inhibits their growth in liquid culture medium. Moreover, glucose uptake and glucose-stimulated oxygen uptake of the promastigotes are significantly inhibited by the antiserum. The results indicate that the antiserum has a profound lethal effect on the *in vitro* propagation of the parasite.

Keywords. *Leishmania donovani*; flagella; antiserum; surface antigens; glucose uptake; oxygen uptake.

Introduction

Leishmania donovani, the parasitic protozoan which causes visceral leishmaniasis or Indian Kala-azar exists in two morphological forms. The flagellated promastigotes are introduced by sandfly vector into the mammalian host and they are then transformed into obligate intra-cellular amastigotes within the phagolysosomes of the host macrophages. Earlier reports indicated that the culture forms of *L. donovani* and *Trypanosoma brucei* were attached to the macrophages by their flagella (Miller and Twohy, 1967; Stevens and Moulton, 1977). On the other hand, Dvorak and Schmunis (1972) reported that while all motile forms of *T. cruzi* epimastigotes actively penetrated macrophages by their flagellar end first, the trypomastigotes did so by their posterior end. In contrast, Chang (1979) observed no preferential orientation of *L. donovani* promastigotes during their entry. Therefore, he concluded that motility of promastigotes and their affinity for the surface of the macrophage were elements of importance in the process of entry. The role of the flagellum during early events of host parasite interaction, such as attachment and entry, is still not clear.

However, when mice were immunized with different subcellular fractions of *T. cruzi* epimastigotes, the flagellar (F) fraction conferred the highest protection against experimental Chagas' disease (Segura *et al.*, 1976). Similarly immunisation with purified flagella from other trypanosomatids, like *Herpetomonas samuelpessoai* and *Crithidia fasciculata*, was also found to be significantly effective against *T. cruzi*

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properties of membrane and cell surface antigens of *Leishmania* promastigotes were extensively studied (Dwyer, 1980; Gottlieb and Dwyer, 1981; Handman *et al.*, 1984; Ramley *et al.*, 1984; Bouvier *et al.*, 1985; Chang and Chang, 1986; Russel and Wilhelm, 1986), neither the biochemical characteristics of *L. donovani* flagellum nor its antigenic properties were carefully examined. In this communication, we have described the isolation and preliminary characterisation of the F fraction of *L. donovani* promastigotes. Antiserum raised against this fraction exhibited both lethal and growth-inhibitory effects on the *in vitro* propagation of the parasite.

Materials and methods

Chemicals

All the biochemicals used were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Brain-heart infusion, and complete and incomplete Freund's adjuvants were products of Difco Laboratories, USA. (^{125}I)-NaI (carrier-free) and (U-^{14}C)-D-glucose (53 mCi/mmol) were purchased from Bhabha Atomic Research Centre, Bombay. Nitro cellulose paper (BA 85; 0·45 μm pore size) was purchased from Schleicher and Schuell, USA. All other chemicals used were of analytical grade.

Parasite culture

L. donovani strain UR6 (Ghosh *et al.*, 1983), a clinical isolate was obtained from Dr. D. K. Ghosh, Indian Institute of Chemical Biology. It was routinely maintained on blood agar slants containing 3·7% brain-heart infusion, 2% whole rabbit blood, 1% glucose and 1·5% agar at 25°C. In liquid medium, whole blood was replaced with 0·1% hemolysed blood. Cells were subcultured at 96 h intervals.

Isolation of flagella and pellicular membrane

The F fraction was prepared following the procedure of Pereira *et al.* (1977) with some modifications. Briefly, cells were harvested, suspended in cold phosphate buffered saline (PBS) and washed twice by centrifugation at 700 *g* for 10 min at 4°C. In all subsequent steps the preparations were maintained at 2–4°C. The pellet was resuspended in 9 volumes of buffer [20 mM Tris-HCl, pH 8; 250 mM sucrose; 3 mM MgCl$_2$; 2 mM phenyl methyl sulphonyl fluoride (PMSF) and 0·5% Triton X-100], homogenised with a tight-fitting Dounce homogenizer (40 strokes) and centrifuged at 700 *g* for 5 min. The supernatant was recentrifuged at 10,000 *g* for 10 min. The resulting pellet was suspended in 5% (w/v) sucrose and the suspension layered over 25% sucrose and centrifuged at 700 *g* for 10 min. The upper 5% sucrose layer was removed and layered over another 25% sucrose solution and the operation was repeated 3 times. The final pellet was obtained by centrifugation of the 5% sucrose layer at 10,000 *g* for 15 min and was suspended in PBS (100 mM NaCl and 50 mM sodium phosphate buffer, pH 7·2) and referred to as F fraction. The

(a total of about 2 mg protein/rabbit) with 2-week intervals, the first injection containing complete Freund's adjuvant and the rest, incomplete Freund's adjuvant. Immunoglobulin fractions were isolated following the method of Michael (1980). Serum was decomplemented by heating at 56°C for 30 min. It was brought to 18% Na_2SO_4 by the addition of 36% Na_2SO_4 solution and centrifuged at 10,000 g for 15 min. The pellet was then dissolved in 0·9% NaCl and reprecipitated in the same way. The final pellet was dissolved in 10 mM phosphate buffer, pH 7·6 to half the initial volume of serum and the solution was dialysed exhaustively against the same buffer. The immunoglobulin G (IgG) fraction was purified by ion-exchange chromatography on a DEAE-cellulose column, equilibrated and eluted with the same buffer.

Gel electrophoresis and immunoblotting

F polypeptides were analysed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) following the method of Laemmli (1970). For immunoblotting experiments the polypeptide components of F and PM fractions were separately resolved by SDS-PAGE (10%) and were transferred electrophoretically to nitrocellulose paper. The nitrocellulose blot was incubated with AS and the antigenic polypeptides detected by [^{125}I]-labelled staphylococcal protein A, following the procedure of Burnette (1981). Protein A (25 μg) was radio-iodinated by Chloramine-T method using carrier-free [^{125}I]-NaI (McConahey and Dixon, 1980). Protein was estimated by Lowry's method (1951), using crystalline bovine serum albumin as the standard.

Oxygen and glucose uptake

Oxygen uptake by promastigotes was measured at 28°C by Warburg's manometric technique (Umbriet et al., 1957). The incubation mixture in a total volume of 3 ml contained $1·5 \times 10^8$ cells (10 mg protein), 150 mM NaCl, 25 mM sodium phosphate buffer pH 7·2, and 5% serum. The reaction mixtures were incubated at 28°C for 1 h before the addition of glucose (5 mM). Uptake of (U-^{14}C)-glucose was measured following the procedure described by Saha et al. (1986); cellulose acetate filters (Millipore Corp., USA) of 1·2 μm pore size were used instead of 0·45 μm pore size filters.

Immunofluorescence

To identify the site (s) of binding of AS, 20 μl of washed cell suspension (10^6 cells) was placed on a glass slide, air dried and incubated with 100 μl (20 times diluted) of either normal serum (NS) or AS for 1 h at 25°C in a moist chamber. Slides were then gently washed twice with PBS and then further incubated for 1 h with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. Slides were finally washed twice with PBS, dried, fixed with 50% glycerol and then examined under Leitz microscope with

revealed by SDS-PAGE (figure 1). The majority of these are in the relative molecular mass (M_r) range of 25–100 kilodalton (kDa) and two of the most abundant ones which seem to be present in equal quantities, are similar in size to the α- and β-subunits of tubulin (54 and 52 kDa).

AS raised against the F fraction interacted with many components of the latter, as attested by the multiple precipitin bands in the double diffusion test (figure 2). It is also observed that distinct precipitin reaction occurs even at AS dilution as high as 8-fold, while there is no detectable band in the case of the undiluted NS.
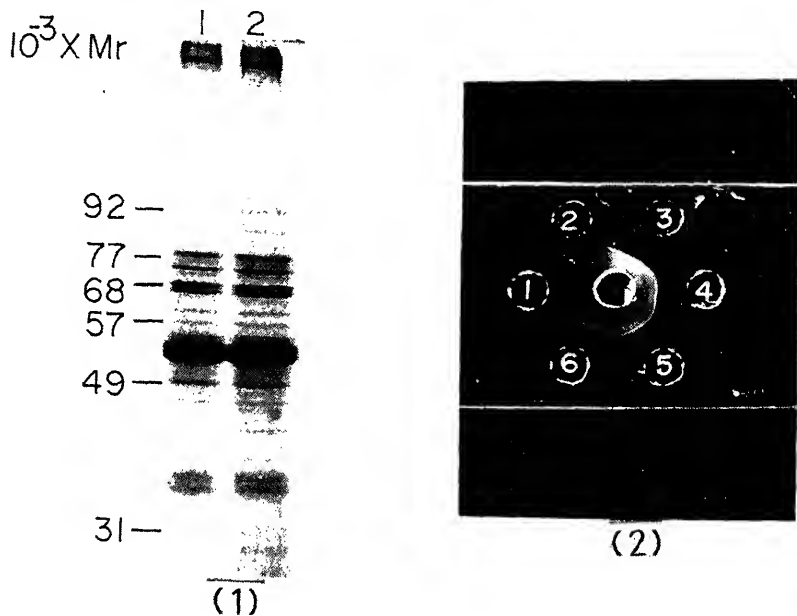
Figures 1 and 2. 1. SDS-PAGE polypeptide profile of F fraction of *L. donovani* promastigotes. M_r markers (numbers on the left) are phosphorlylase b, transferrin, bovine serum albumin, pyruvate kinase, fumarase and carbonic anhydrase. Lane 1, 50 μg and lane 2, 100 μg of F fraction. 2. Ouchterlony double diffusion analysis of antiserum to F fraction of *L. donovani* promastigotes. Central well, 50 μl of F fraction (2 mg/ml); wells 1 and 2, 50 μl of undiluted and 2 times diluted NS; wells 3–6, 50 μl of undiluted, 2, 4 and 8 times diluted AS, respectively.

Using AS, further characterization of the F fraction was carried out by immunoblot analysis. Figure 3 shows that in both F and PM fractions, there are a number of polypeptides in the M_r range of 55–48 kDa which are strongly reactive with AS. Five other polypeptides of the F fraction (73, 70, 66, 62 and 33 kDa) are also found to interact with AS. The 66 and 33 kDa polypeptides are also present in the PM preparation. Therefore, only 3 antigens (73, 70 and 62 kDa) appear to be flagellum-specific
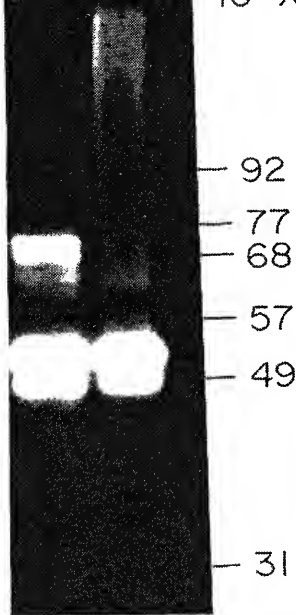
Figure 3. Western blot analysis of immunogenic F polypeptides of *L. donovani* promastigotes. Numbers on the right are M_r of marker proteins (as in figure 1). Lane 1, 20 μg of F protein; line 2, 20 μg of PM protein.

donovani promastigotes were completely agglutinated and lost their motility ~tructural integrity in the presence of 1% heat-inactivated AS (figure 4b). Under r conditions, even 10% heat-inactivated NS was completely ineffective ~ 4a).

~le 1 shows the effect of heat-inactivated AS on the growth of *L. donovani* ~astigotes. A nearly 30-fold increase in the number of viable cells was observed ~72 h when promastigotes were cultured alone or in the presence of heat-~vated normal serum (NS). Under identical conditions, less than 10% of the ~lated promastigotes remained viable in the presence of AS up to 1:200 dilution. ~dition, this was also the minimum concentration of AS needed for appreciable ~tination of promastigotes in culture (table 1). At 1:400 dilution of AS, proma-~es were not agglutinated but their growth was completely inhibited. Therefore, ~s both lethal and growth-inhibitory effects on *L. donovani* promastigotes.

~ty to sixty percent inhibition in glucose uptake was observed when promasti-~were treated with AS (figure 5). Along with glucose uptake, glucose-stimulated ~ration was also reduced by a similar extent (figure 6). Neither glucose uptake ~lucose-induced respiration was affected in the presence of NS.

~munofluorescence experiments revealed that AS not only binds to the flagellum
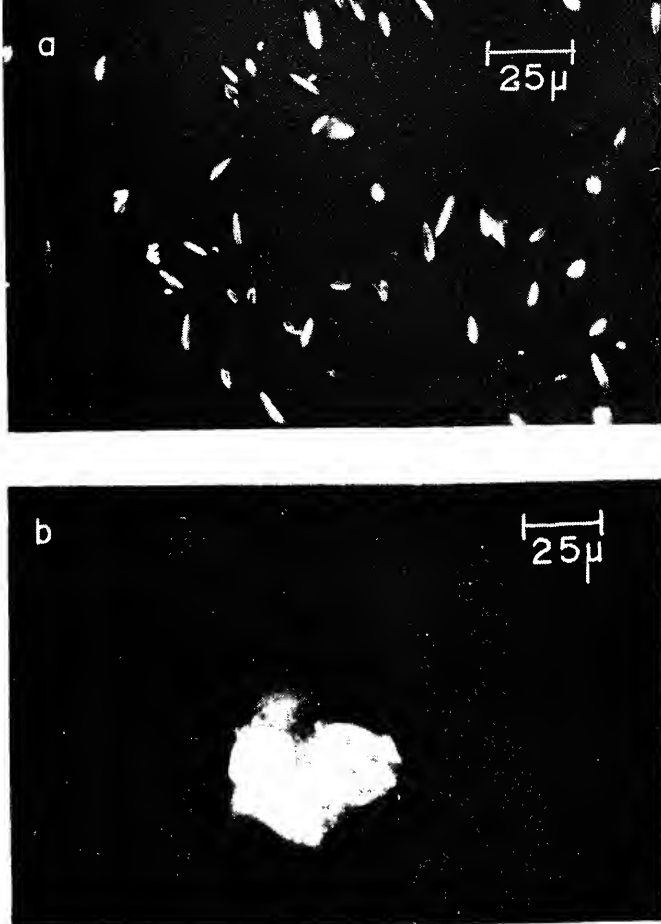
Figure 4. Agglutination of *L. donovani* promastigotes in the presence of antiserum. Promastigotes were preincubated for 30 min at 25°C with (a) 10% NS and (b) 1% AS.

Discussion

The two predominant polypeptides of the flagellum of *L. donovani* are tentatively identified as the subunits of tubulin on the basis of their size and abundance. Even though rigorous characterisation has not been attempted in the present studies, this conclusion is consistent with previous findings in the case of other hemoflagellates (Whitman *et al.*, 1972; Pereira *et al.*, 1977). Moreover, Dwyer (1980) has reported that the PM of *L. donovani* is rich in tubulin. The results of the immunoblot experiment, showing strong immunoreaction with polypeptides in the M_r range of 55 and 48 kDa of both F and PM fractions, support the conclusion that tubulin may be the major component of the *L. donovani* flagellum.

Additions	dilution	(μg/ml)	Agglutination[a]	No. of promastigotes[b] $\times 10^{-5}$
None	–	–	Nil	35 ± 7[c]
NS	1:10	–	Nil	33 ± 8
AS	1:10	–	+ + +	0·1
AS	1:100	–	+ +	0·1
AS	1:200	–	+	0·1
AS	1:400	–	±	4 ± 1
AS	1:800	–	Nil	37 ± 6
NS-IgG	–	500	Nil	36 ± 8
AS-IgG	–	500	+ + +	0·1
AS-IgG	–	250	+ +	0·1
AS-IgG	–	100	±	10 ± 2
AS-IgG	–	50	Nil	35 ± 6

[a] + + +, + +, + and ± indicate heavy, medium, light and doubtful agglutination.
[b] Promastigotes' (1×10^5/ml) were cultured in liquid blood medium in the presence of AS or IgG fraction. Cells were counted after 72 h at 25°C.
[c] Numbers are mean ± SD of 5 independent experiments.
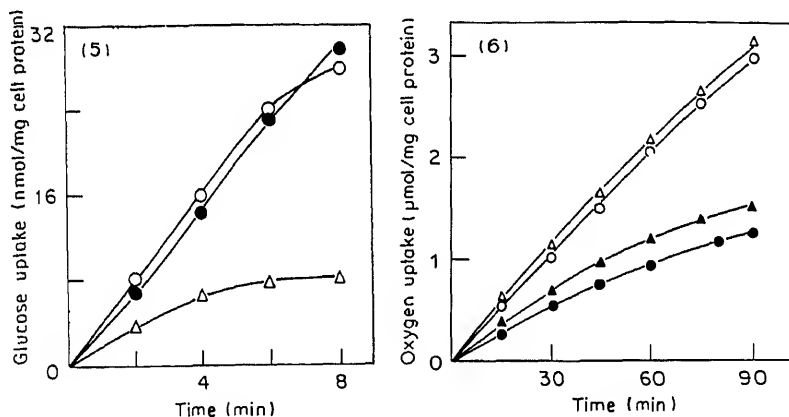
Figures 5 and 6. 5. Effect of antiserum on glucose uptake by *L. donovani* promastigotes. (○), Promastigotes in the absence of serum; (●), promastigotes + 5% NS; (△), promastigotes + 5% AS. 6. Effect of AS on glucose-induced oxygen uptake by *L. donovani* promastigotes. (●), Promastigotes in the absence of glucose and serum; (○), promastigotes + 5 mM glucose; (△), promastigotes + 5 mM glucose + 5% NS; (▲), promastigotes + 5 mM glucose + 5% AS.

inactivated serum agglutinated but could not kill the promastigotes (Pearson and Steigbigel, 1980). Activation of the complement cascade by classical or alternative pathway was shown to be responsible for this lethal effect (Pearson and Steigbigel 1980; Mosser and Edelson, 1984). In all experiments, we have used heat-inactivated immune serum. Therefore, *Leishmania*-specific antibodies in the immune serum are directly responsible for its lethal and growth-inhibitory effect. The possibility that
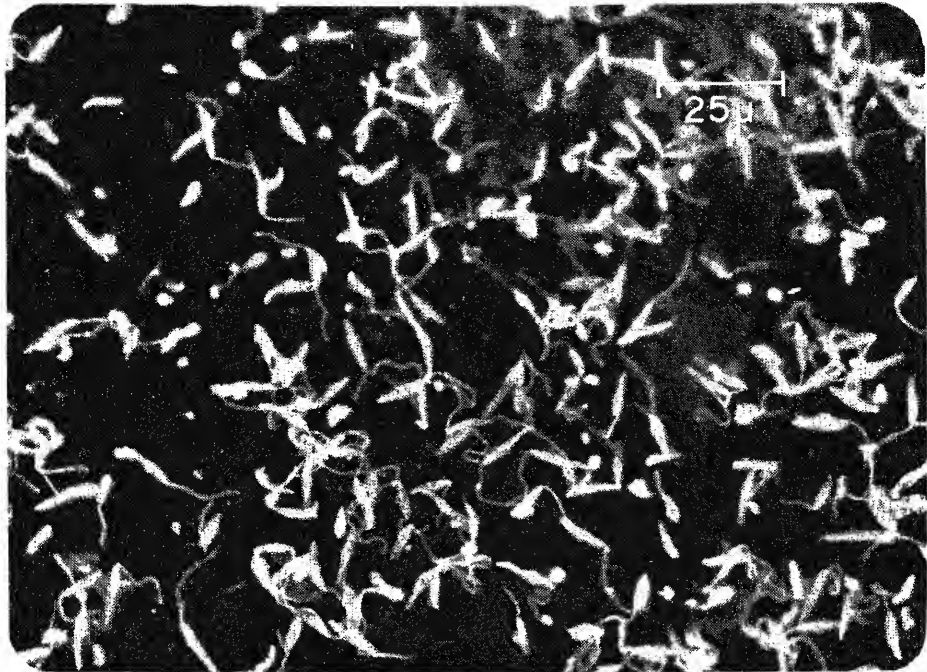
Figure 7. Indirect immunofluorescence staining of *L. donovani* promastigotes with AS to F fraction.

purified IgG was found to have similar effects. The lethal effect of immune serum was apparent only when the promastigotes were agglutinated (table 1).

Selective transport of metabolites is a characteristic property of all biological membranes. In *Leishmania* species, the existence of a glucose transport system was reported by Schaffer and Mukkada (1976) and by Zilberstein and Dwyer (1984, 1985). Since glucose could be metabolized rapidly by *Leishmania* promastigotes, results of uptake studies with (U-^{14}C)-glucose might not reflect the true kinetics of active transport across the cell membrane. However, impairment of membrane function was evident from significant reduction in glucose uptake by promastigotes in the presence of AS (figure 5). A similar reduction in glucose induced oxygen uptake (figure 6) therefore appears to be a secondary phenomenon and is probably due to reduced glucose uptake by *L. donovani* promastigotes. Immune serum binds evenly over the entire surface of the promastigote (figure 7). This is rather expected as 4 antigens are shared by the F and PM fractions of *L. donovani* promastigotes (figure 3).

Molecular interactions at the host-parasite interphase are critical for obligatory intracellular parasites such as *Leishmania*. Mosser and Edelson (1984) first proposed that in the presence of serum, enhanced binding of *Leishmania* promastigotes to murine resident peritoneal macrophages is mediated by complement protein C_3. In

ts indicate that even 0·25 mg/ml of AS-Fab can inhibit *L. donovani* promastigote
ing to macrophages to the same extent. Both the glycolipid and the gp63 are
ly soluble in detergents like Triton X-100, NP-40 and octylglucoside. Since the F
ion used in the present study was isolated from the particulate fraction
tially free from the triton X-100 soluble material, it is unlikely that the
ition of parasite binding by AS-Fab is entirely due to the presence of Fab
ents derived from antibodies directed against contaminating glycolipid or gp63
e F fraction. This observation raises the possibility that parasite antigen (s) other
the glycolipid or gp63 may also be involved in host-parasite interaction.

humans, the only prophylactic immunisation strategy with any success against
manial diseases needed controlled induction of a cutaneous lesion with low
s of *Leishmania tropica* (Greenblatt, 1980). Experimentally, several immunisation
egies were tried against cutaneous leishmaniasis and in all cases only partial
ection was achieved. These included immunisation with ultrasonicated or γ-
liated promastigotes (Preston and Dumonde, 1976; Howard *et al.*, 1982), crude
gen-antibody complex (Handman *et al.*, 1977) and affinity-purified *Leishmania*-
ific glycolipid (Handman and Mitchel, 1985). The results presented here clearly
onstrate the profound lethal effect of immune serum on *L. donovani* promasti-
s. At present it is not clear from our results whether immunoglobulins directed
ist F antigens or those against membrane antigens or both are responsible for
lethal effect. Therefore, a thorough and careful study is needed to evaluate the
unoprophylactic potential of cell surface antigens of *L. donovani* promastigotes.

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Induction of glutathione S-transferase in the castor semilooper, *Achaea janata* (Lepidoptera, Noctuidae) following fenitrothion treatment

V. B. YADWAD and V. L. KALLAPUR*

Department of Studies in Zoology, Karnatak University, Dharwad 580 003, India

Abstract. Glutathione S-transferase activity was determined in the lepidopteran insect species, *Achaea janata*, during larval, pupal and adult stages following treatment with sublethal and lethal doses of fenitrothion. Both doses of insecticide produced significant induction of enzyme activity. The rate of induction of enzyme activity was not significantly different in insects that received sublethal and lethal doses of insecticide. Enzyme activity in the different stages of insecticide-treated insects was in the order pupa > adult > larva. However, the inducing effect of the insecticide was higher in larvae than in pupae and adult. In the absence of induction, the level of enzyme was as much as 3 times higher in midgut tissue than in carcass. In larvae treated with sodium barbitone along with fenitrothion, the knock-down effect of the insecticide was delayed. This was attributed to the increased induction of glutathione S-transferase in the larvae treated with sodium barbitone. The level of reduced glutathione, a rate-limiting factor in the induction of glutathione S-transferase, changed in a cyclic manner in insecticide-treated larvae.

Keywords. Induction; fenitrothion; glutathione; glutathione S-transferase.

Introduction

Conjugation of xenobiotics with reduced glutathione (GSH), catalyzed by gluta-thione S-transferase (GSH S-transferase), is an important physiological process in the elimination of toxic substances from the body. The role of GSH S-transferase is considered to be an important mechanism in insect resistance to organophosphate (OP) insecticides (Oppenoorth *et al.*, 1977; Motoyama and Dauterman, 1980). The presence of this enzyme has been reported in resistant and susceptible strains of the house fly (Lewis, 1969; Lewis and Sawicki, 1971; Motoyama and Dauterman, 1980) and in the blowfly (Hughes and Devonshire, 1982). It has been demonstrated that in the house fly phenobarbital (Ottea and Plapp, 1981) and several insecticides induce the activity of GSH S-transferase (Hayaoka and Dauterman, 1982). DDT was found to be the most active in inducing transferase activity and it was also found that flies with induced GSH S-transferase were more tolerant to several OP insecticides (Motoyama and Dauterman, 1980). Lepidopteran species, which include notorious pests of agricultural crops have not been investigated in respect of the induction of GSH S-transferase following OP treatment. In the present study the activity of GSH S-transferase in the castor semilooper *Achaea janata* L. was determined following treatment with lethal and sublethal doses of fenitrothion, an OP insecticide.

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*To whom all correspondence should be addressed.
Abbreviations used: GSH, Reduced glutathione; OP, organophosphate; DCNB, 3,4-dichloronitrobenzene; MFO, mixed function oxidases.

Materials and methods

Various stages of the insect, *A. janata*, employed in this study were obtained from the laboratory culture maintained at 26°C with 16:8 L:D photoperiod. Precisely aged larvae (24 h after the 4th moult), 72 h-old pupae and 48 h-old moths were used. All solvents used were of analytical grade (Glaxo, Bombay). 3,4-Dichloronitrobenzene (DCNB) and sodium barbitone were obtained from Fluka, West Germany and GSH from Sigma Chemical Co., St. Louis, Missouri, USA. Pure technical grade fenitrothion was a gift sample from M/s, Rallis, Bombay.

Bioassay and induction experiments

Lethal dose (LD_{50}) of fenitrothion was determined by analyzing acute lethality test data by probit analysis (Finney, 1964). Thirty and 10 μg of fenitrothion were used as lethal and sublethal dose respectively for 5th instar larvae. The same concentration of insecticide were used for pupae and adult moths. Five μl of acetone solution of the insecticide were applied topically to the dorsal side of the larva, pupa and adult. In the case of adult moth the scales of the dorsal side of the abdomen were removed before the application of the insecticide. Insects treated with lethal dose of fenitrothion exhibited excitation, tremors and paralysis, and finally died. The insects treated with lethal dose showed early stage of prostration at 4·5 h, while in the case of larvae treated with sublethal dose the excitation was noted 5 h after the treatment. In all our experiments the insects showing early prostration and excitation were used. However, in the case of pupae, the insects were used 5 h after the treatment since no symptoms of insecticide poisoning were observed.

Preliminary observations on the response of larvae to sodium barbitone indicated that 10 μg of the compound produced no mortality in the treated group. In the present study 10 μg of sodium barbitone dissolved in 5 μl distilled water were injected using a Hamilton microsyringe. Insects treated topically or injected with an equal volume of acetone/water served as controls. In all these experiments groups of 5 insects were used. Each experiment was repeated 3 times.

After the appropriate time, the insects were cut into small pieces before being homogenized. For the collection of larval midgut and carcass, the larvae were dissected in ice cold insect Ringer's solution. The alimentary canal was separated and removed from the larval body. The midgut was isolated from the alimentary canal. The tissue remaining after complete removal of the digestive tract was designated carcass. Each tissue was homogenized in ice cold glass distilled water using a Potter-Elvehjem glass homogenizer with teflon pestle. The homogenate was centrifuged at 8000 g to separate mitochondria nuclei and cell debris. The supernatant was used as the source of enzyme immediately. All operations were carried out at 2–4°C. Protein was determined according to the method of Lowry *et al.* (1951).

GSH S-transferase activity was determined spectrophotometrically according to the method described by Motoyama and Dauterman (1975). Incubation medium contained in a final volume of 3 ml, 16 mM GSH in 1 ml Tris-HCl buffer, pH 8·5

specified in the present study.

GSH was determined using 0·05 M sodium nitroprusside, at 525 nm (Allport and Keyser, 1957).

Incorporation of labelled leucine

Five μl of solution containing 0·05 mCi (specific activity 335 mCi/mmol) of [U-^{14}C] leucine was injected into the hemocoel of 5th instar larvae using a Hamilton micro-syringe. Three h after injection (time required for maximum incorporation), different tissues were collected by dissecting insects in cold insect saline. Incorporation of labelled leucine into proteins was determined by a filter paper disc method (Mans and Novelli, 1962). The radioactivity was measured by using a Beckman liquid scintillation counter.

Results

Both sublethal and lethal doses of fenitrothion caused significant increase in the enzyme activity. In the absence of induction, the level of enzyme in midgut tissue was as much as 3 times higher than that in carcass. On the other hand the induction of the enzyme in insecticide-treated larvae was considerably higher in carcass than that in midgut (table 1). The results also revealed that the rate of induction of enzyme activity was not significantly different in larvae that received sublethal and lethal doses.

Table 1. Induction of GSH S-transferase activity by lethal and sublethal doses of fenitrothion in the midgut and carcass of 5th instar castor semilooper larvae.

| Dose | Tissue | nmol DCNB conjugated/min/mg protein | | Treated/ control[a] |
		Control	Treated	
Lethal	Midgut	60·8 ±2·56	98·02±15·92*	1·60
	Carcass	20·67±1·39	48·55±1·64**	2·30
Sublethal	Midgut	55·1 ±2·75	115·4±6·15**	2·00
	Carcass	19·67±1·06	51·0±2·45**	2·6

All values are mean±SE of mean of 5 experiments using 5 insects, in each experiment.
[a]Ratio of activity in insecticide-treated larvae to that in untreated larvae (n=3).
*Significant difference from control, $P<0·05$; **$P<0·001$ (Student's t test).

The induction of GSH S-transferase was time-dependent. The maximum activity in midgut and carcass was obtained at 8 and 6 h, respectively and the level was maintained upto 14 h. Thereafter, the enzyme level declined to the normal level in both tissues (figure 1). The uninduced levels of enzyme in 3 developmental stages of the insect were in the order pupa > adult > larva. However, it was noted on the basis of the ratios of activity in insecticide-treated insects to that in untreated ones that the inducing effect of the insecticide was higher in larvae (2·5) than in pupae (1·5) and
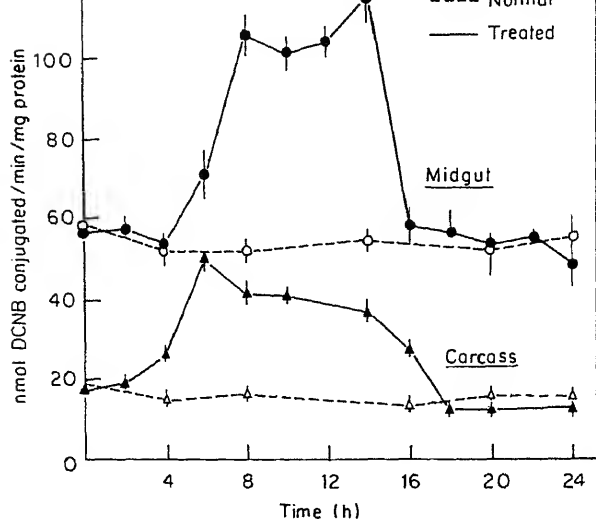
Figure 1. Induction of GSH S-transferase activity in midgut and carcass of 5th instar castor semilooper larvae by sublethal dose of fenitrothion.

Table 2. Effect of sublethal dose of fenitrothion on GSH S-transferase activity in the 3 developmental stages of castor semilooper.

Stage	n mol DCNB conjugated/ min/mg protein		Treated/ control[a]
	Untreated	Treated	
5th instar larvae	184·55 ± 16·1	464·33 ± 20·06	2·5
Pupae	395·8 ± 14·95	594·6 ± 13·98	1·5
Adult	240·35 ± 17·37	389·5 ± 18·35	1·6

All values are mean ± SE of 5 experiments.
[a]Ratio of activity in insecticide-treated insect to that in control insect (n = 3).

et al., 1961). This was tested in 5th instar larvae by injecting sodium barbitone (10 μg/ larva) along with fenitrothion. The results reveal that the knock-down effect of the insecticide was significantly delayed in the sodium barbitone treated larvae (table 3). The induction of GSH S-transferase in sodium barbitone treated larvae began after 4 h and reached the maximum at 18 h from the time of injection of the compound (figure 2).

The level of GSH may be rate-limiting factor in the induction of GSH S-transferase activity. GSH content of midgut and carcass of 5th instar larvae after treatment with sublethal dose of insecticide is presented in table 4. The tissues responded differently to the treatment. The results reveal that the GSH content of the midgut showed a cyclic response. The GSH level was lower than normal at 4 h and higher at 8 h and

toxicity in 5th instar castor semilooper larvae.

| Treatment (μg/larva) | LT$_{100}$(h) | |
	Fenitrothion	Sodium barbitone and fenitrothion
20	8·0 ± 0·35	20·0 ± 0·62**
30	6·3 ± 0·56	12·0 ± 1·30*
35	5·0 ± 0·30	11·0 ± 0·69**
40	4·0 ± 0·48	10·0 ± 0·85**

All values are mean ± SE of mean of 3 experiments.
*Significant difference from fenitrothion, $P < 0.05$; **$P < 0.001$.
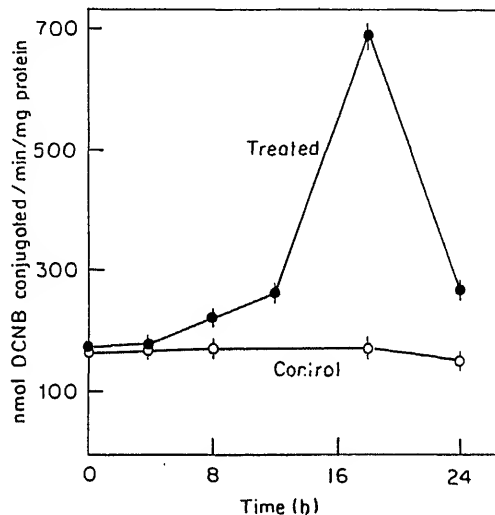
Figure 2. Induction of GSH S-transferase activity by barbitone in the 5th instar castor semilooper larvae.

Table 4. GSH and GSH S-transferase in midgut and carcass of 5th instar castor semilooper larvae following treatment with sublethal dose of fenitrothion.

| Time after treatment (h) | GSH (μg/mg protein) | | GSH S-transferase (n mol DCNB conjugated min/mg protein) | |
	Midgut	Carcass	Midgut	Carcass
00	55·0 ± 2·8[a]	45·1 ± 2·0[a]	55·4 ± 2·6[a]	17·0 ± 1·12[a]
04	35·2 ± 2·0[b]	45·7 ± 2·0[a]	56·7 ± 2·5[a]	25·2 ± 1·8[b]
08	70·7 ± 3·6[c]	35·4 ± 1·2[b]	110·1 ± 3·2[b]	42·5 ± 2·6[c]
12	40·3 ± 2·0[bd]	32·1 ± 2·6[b]	105·2 ± 3·0[b]	40·9 ± 2·0[c]
16	70·8 ± 4·2[ce]	30·0 ± 1·0[b]	60·6 ± 2·8[a, c]	27·6 ± 1·96[bd]
20	60·5 ± 3·0[ce]	26·2 ± 1·8[b]	56·6 ± 2·0[a, c]	18·1 ± 1·3[a, e]
24	57·4 ± 2·8[adef]	45·3 ± 3·0[ac]	48·3 ± 2·0[a, c]	18·0 ± 1·45[a, e]

higher at 8 and 12 h, and lower at subsequent times until 24 h. Interestingly, carcass GSH showed no such cyclic response during the experimental period. Significant reduction in the GSH level was observed only at 8 h following the treatment. The GSH S-transferase activity, on the other hand, was significantly higher at 4 and 8 h, and the level was maintained upto 12 h from the time of treatment. At 16 h the activity was significantly lower than that at 12 h but still higher than normal. Enzyme activity decreased further and was not significantly different from normal at 20 and 24 h.

Incorporation of labelled leucine by midgut and carcass following treatment with sublethal dose of insecticide was significantly higher than that in untreated larvae (table 5).

Table 5. Effect of sublethal dose of fenitrothion on the incorporation of $[U-^{14}C]$-leucine into proteins of the 5th instar *A. janata*.

	[U-^{14}C]-Leucine incorporated	
Tissues	Control	Treated
Midgut	6700 ± 100	9000 ± 200*
Carcass	9900 ± 200	13300 ± 200*

All values are mean$\pm$SE of mean of 5 experiments.
*Significant difference from control, $P < 0.01$.

Discussion

The present study has demonstrated that both fenitrothion and sodium barbitone induce GSH S-transferase activity in the castor semilooper. This effect is similar to that reported in the case of dipteran insects (Motoyama and Dauterman, 1980; Ottea and Plapp, 1981; Hayaoka and Dauterman, 1982). It has been claimed that high GSH S-transferase activity and GSH are necessary components of the GSH S-transferase system for the detoxification of OP insecticides (Motoyama and Dauterman, 1980) and industrial pollutants (Chatterjee and Bhattacharya, 1984) in various animal systems. In the present study the GSH content of both midgut and carcass varied in response to fenitrothion treatment and could not account for the increased level of enzyme activity after treatment. This phenomenon has also been recorded in other insects (Motoyama and Dauterman, 1980). It has been suggested that GSH S-transferase itself acts as a binding protein and the enzyme is known to bind diverse groups of chemicals including carcinogens (Motoyama and Dauterman, 1980). The question whether the GSH S-transferase of the castor semilooper binds to fenitrothion needs further investigation. Studies using other insects have shown that phenobarbitone induces GSH S-transferase and mixed function oxidases (MFO) (Wilkinson and Brattsten, 1973; Ottea and Plapp, 1981; Hayaoka and Dauterman, 1982). However, there are wide-spread reports that phenobarbitone is not

administered (table 3). The knock-down effect of fenitrothion, when administered alone, occurs within 8 h from the time of application of the insecticide. It appears that the reduced toxicity of fenitrothion in larvae that also received barbitone may be due to the induction of GSH S-transferase as well as that of MFO. That increased MFO activity is usually associated with an increase in protein synthesis is shown by the increased incorporation of labelled amino acids into proteins (Agosin *et al.*, 1966; Kato *et al.*, 1966). It is possible that the increased protein synthesis as evident from the increased incorporation of labelled leucine into protein, following fenitrothion treatment (table 5), indicates such`a response of the castor semilooper to the insecticide.

The larval midgut showed higher GSH S-transferase activity than the carcass. This result is in contrast with that reported for adult American cockroach in which carcass exhibited higher enzyme activity (Shishido *et al.*, 1972). This difference in response may be due to differences in adaptive strategies employed by each insect species in response to exposure to xenobiotics. The larvae of castor semilooper are a pest of agricultural crops, whereas the cockroach is more of a domestic pest. The rate of induction of enzyme activity was higher in carcass than in midgut. Differences in the rate of induction of enzyme activity may be due to the intrinsic level of GSH S-transferase. Induction was much more in strains of housefly which have a low uninduced level of GSH S-transferase than in those which have a high uninduced level of enzyme activity (Hayaoka and Dauterman, 1982).

The rate of induction of GSH S-transferase was significantly higher in 5th instar larvae than in pupae and adults. This is expected since the larvae are more vulnerable to xenobiotics originating either from the host plants or from insecticides sprayed on them.

Acknowledgement

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Cholesteryl-(2'-hydroxy)-ethyl ether—A potential cholesterol substitute for studies in membranes

ANIL K. LALA* and S. NANDA KUMARI

Biosciences and Engineering Group, Department of Chemistry, Indian Institute of Technology, Powai, Bombay 400 076, India

Abstract. The yeast sterol auxotroph GL-7, which grows well on ergosterol and cholesterol, was used to study the ability of cholesteryl-(2'-hydroxy)-ethyl ether to substitute for cholesterol. In this compound the 3β-hydroxyl group of cholesterol is replaced by ethylene glycol and the resulting ether still retains the amphiphilic character of cholesterol. Cholesteryl-(2'-hydroxy)-ethyl ether was found to support the growth of GL-7 as effectively as cholesterol. Crystal violet permeability and membrane order parameter determined using a spin label were similar for cells grown on these sterols. The ability of such ethylene glycol derivatives to substitute for cholesterol in both artificial and natural membranes should help in designing suitable spacers through which molecules can be linked to cholesterol without affecting the normal function of cholesterol in membranes. This in turn should prove useful in studies with surface-modified liposomes.

Keywords. Cholesteryl-(2'-hydroxy)-ethyl ether; yeast sterol auxotroph; membranes; anchor molecule.

Introduction

Cholesterol is an integral component of various biological membranes. It also affects the fluidity of biological membranes and is found to be associated with various membrane-associated processes like permeability, activity of membrane-bound enzymes and receptors, endocytosis and immune response (Demel and De Kruyff, 1976; Yeagle, 1985). The interaction of cholesterol with phospholipids and proteins is well documented (Yeagle, 1985). A planar ring system (Demel *et al.*, 1972), an isoctyl chain (Suckling *et al.*, 1979; Bloch, 1983), a C_5–C_6 double bond (Ranadive and Lala, 1987) and a 3β-hydroxyl group at C_3 (Demel and De Kruyff, 1976) have been considered essential for optimal interaction of cholesterol with other membrane components. We have for some time been involved in examining the role of hydroxyl group of cholesterol in membranes (Lala, 1981) and have recently reported that a free hydroxyl group in cholesterol is not necessary for the normal cholesterol-associated properties observed in model membranes (Demel *et al.*, 1984). This study involved the use of various alkyl ethers of cholesterol and revealed that increasing the hydrophobic bulk of the ether *i.e.*, going from cholesteryl methyl ether to cholesteryl butyl ether, decreases its ability to condense membranes as judged by monolayer, differential scanning calorimetric and glucose permeability studies on liposomes. Nevertheless cholesteryl methyl ether substituted very well for cholesterol. Interestingly the introduction of a hydroxyl group in place of the terminal methyl

hydroxy)-ethyl ether (CH-OEG), which also substitutes for cholesterol.

We have also reported that cholesteryl methyl ether is as effective as cholesterol in supporting the growth of the *Saccharomyces cerevisiae* double mutant GL-7 (Lala *et al.*, 1979). This yeast mutant is an effective sterol auxotroph (Gollub *et al.*, 1977) and has been successfully used to study the role of sterol in natural membranes (Buttke and Bloch, 1981; Nanda Kumari *et al.*, 1982; Bloch, 1983). We reported here our studies with the novel compound, CH-OEG using GL-7.

Materials and methods

Cholesterol was obtained from SRL, Bombay and crystallised twice from methanol before use. CH-OEG (figure 1) was prepared as reported earlier (Demel *et al.*, 1984). Both sterols were purified by high performance liquid chromatography (HPLC) and found to be homogeneous. HPLC analysis was carried out on a Dupont 8800 system or a Shimadzu LC-4A system using an RI or UV detector. Methanol or methanol: water (98:2) was used as mobile phase. Cholesterol appeared at 13·6 min and CH-OEG at 15 min when methanol was the mobile phase. Increasing water in the mobile phase or gradient elution did not lead to improved separation of the two sterols.

The *S. cerevisiae* mutant GL-7 was grown on these sterols. Growth of the cells and isolation of nonsaponifiable lipid extract were carried out as described earlier (Nanda Kumari *et al.*, 1982). HPLC analysis of the extract from cells grown on CH-OEG indicated no trace of cholesterol. Thin-layer chromatographic (TLC) analysis was also carried out on silica gel G coated plates using 15% ethyl acetate in benzene as developing solvent system. For NMR spectroscopic analysis, the nonsaponifiable lipid extract of cells grown on CH-OEG (2 L batches) was subjected to TLC and a broad band between R_f's 0·3 and 0·7 was cut and extracted with chloroform: methanol (2:1). The solvent was then removed and the NMR spectrum of the residue in $CDCl_3$ was recorded on a Bruker 270 MHz spectrometer.

Crystal violet was obtained from Glaxo, Bombay. A fresh 18 h GL-7 culture was shaken well. Three ml aliquots were taken in glass centrifuge tubes. Crystal violet was added as a solution in ethanol (30 μl, 0·5 mg/ml) to each tube, and the tubes incubated at room temperature (30°C) for 10 min. The cells were centrifuged out and the absorbance at 590 nm of the supernatant was determined. The crystal violet index was obtained by dividing the mean absorbance at 590 nm of the supernatant of a culture grown on cholesterol by the mean absorbance at 590 nm of the supernatant of the test culture. A correction factor was applied for the difference in the number of cells.

7-Doxyl stearic acid was prepared by published procedure (Jost *et al.*, 1971) Electron spin resonance spectra were recorded on a Varian E-12 spectrometer. 7-Doxyl stearic acid was incorporated in GL-7 cells grown on cholesterol and CH-OEG and order parameter determined from the ESR spectra as reported earlier (Lees *et al.*, 1979).

Results and discussion

Figure 1. Structure of CH-OEG.
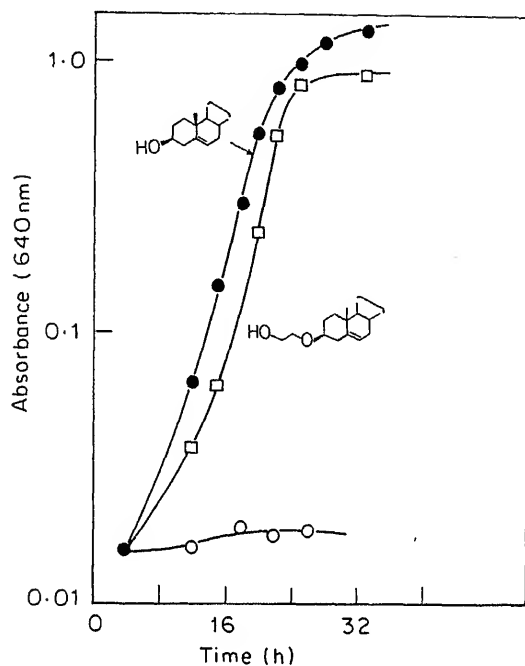
Figure 2. Growth curve of GL-7 cells grown on cholesterol (●), CH-OEG (□), and in the absence of sterol (○).

the sterols were found to be quite effective in supporting growth over several cycles (figure 2). It has been reported that cholesterol is not metabolically transformed while supporting growth of GL-7 (Lala *et al.*, 1979; Buttke and Bloch, 1981). To confirm that CH-OEG is not metabolized during growth, the nonsaponifiable lipid extract was analysed by reversed phase HPLC. The analysis confirmed the identity of the compound and showed no trace of cholesterol. To further confirm the identity of CH-OEG, NMR spectroscopy was used. This method has been used in the past to identify other sterols which support the growth of GL-7 (Nanda Kumari *et al.*, 1982). Although it is much less sensitive than HPLC, NMR spectroscopy is an independent method for sterol identification. Cells were grown in bulk on CH-OEG and the sterol fraction of the nonsaponifiable extract of the cells was isolated. The NMR spectrum of the sterol fraction was obtained and found to match that of authentic CH-OEG clearly indicating that this compound had not undergone metabolic transformation while supporting the growth of GL-7.

It is well known that increasing the amount of cholesterol in membranes leads to

violet and membrane order parameter were determined for cells grown on these sterols. Crystal violet is a cationic dye and has been used to determine the permeability of yeast mutants (Bard et al., 1978). The data given in table 1 clearly indicate similarity in crystal violet permeability for GL-7 cells grown on cholesterol and those grown on CH-OEG. Spin labelled probes like 7-doxyl stearic acid have been quite useful in assessing the degree of order in membranes, which increases with increasing concentration of cholesterol (Schreier et al., 1978; Lees et al., 1979). The membrane order parameter determined for cells grown on cholesterol and that for cells grown on CH-OEG were also found to be similar (table 1). In order to see the effect of the two sterols on artificial membranes, order parameter for egg phosphatidyl choline (PC): cholesterol and PC:CH-OEG vesicles was also determined. The order parameter values for the two vesicle preparations were also found to be similar (table 1).

Table 1. Crystal violet permeability and membrane order parameter $[S_{7DS}]$- for GL-7 cells grown on cholesterol and on CH-OEG.

Experiment	Cholesterol	CH-OEG
Crystal violet permeability for cells	1.00 ± 0.02	0.91 ± 0.01
$[S_{7DS}]$ for cells	0.67 ± 0.02	0.68 ± 0.04
$[S_{7DS}]$ for PC:sterol vesicles	0.64 ± 0.03	0.63 ± 0.02

Values are mean $\pm$ SD of at least 3 determinations.
$[S_{7DS}]$ for PC:sterol vesicles (33 mol% sterol) included for comparison.

The GL-7 growth data and the preliminary studies on membrane-associated properties of these cells indicate that CH-OEG is quite effective in simulating the role of cholesterol. This confirms similar conclusions made on the basis of artificial membrane studies (Demel et al., 1984). The ability of CH-OEG to substitute for cholesterol indicates that it should be possible to synthesise cholesterol analogues with the hydroxyl group substituted by other groups without affecting the normal function of cholesterol in membranes. Such chemical modifications are important for the use of cholesterol as an anchor molecule for attaching membrane surface-active agents like sugars and antibodies. Earlier studies along these lines have involved the use of carbamate links for attaching sugars (Slama and Rando, 1980) and fluorescent probes (Alecio et al., 1982) and ester links for attaching proteins (Kinsy et al., 1983). The use of these functional groups, specially cholesteryl esters, is likely to perturb the membrane and thus affect the normal function of cholesterol in membranes. For effective liposome targeting and related studies it will be desirable to have cholesterol analogues with spacers which would permit convenient attachment of molecules of interest and still retain the normal membrane-associated properties of cholesterol. The use of cholesteryl ethers recently in the synthesis of cholesterol analogues carrying β-aminogalactose to study effects of surface modification on aggregation of phospholipid vesicles (Wu et al., 1981) is encouraging. The use of ethylene glycol based ethers of cholesterol as membrane anchor sites thus has considerable potential

Acknowledgements

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Purification and properties of trehalase from monkey small intestine

S. SANKER and S. SIVAKAMI*

Department of Life Sciences, University of Bombay, Vidyanagari, Santacruz (East), Bombay 400 098, India

Abstract. Brush border membrane trehalase was purified from monkey small intestine by a procedure which includes solubilisation by Triton X-100, ammonium sulphate fractionation, and chromatography on DE-52 and hydroxyapatite. The purified enzyme had a specific activity of 11 units/mg protein and was purified 140-fold. The enzyme showed a single protein band on polyacrylamide gel electrophoresis. It had a K_m value of 17·4 mM for trehalose and a V_{max} of 1·33 units. Sucrose and Tris acted as competitive inhibitors of the enzyme.

Keywords. Trehalase; intestinal brush border membrane; hydrophobic protein.

Introduction

Trehalase (EC 3·2·1·28) is a disaccharidase present in the intestinal brush border membrane along with sucrase, maltase, glucoamylase and lactase. Though the enzymic properties and membrane organisation of sucrase and glucoamylase have been studied in detail, there is relatively little information on trehalase. The precise function of trehalase in the brush border membrane is not known. A possible role in glucose transport is suggested (Sacktor, 1968). Purification of trehalase from the intestine of rat and rabbit has been reported (Sasajima et al., 1975; Galand, 1984; Yokota et al., 1986). Trehalase from the brush border membrane of the kidney has been studied in greater detail than the intestinal enzyme where it appears to exist in multiple molecular forms (Nakano and Sacktor, 1985). In this paper, we report a method for the purification of trehalase from the brush border membrane of the monkey small intestine and some of the properties of the enzyme.

Materials and methods

Assay of enzyme

Trehalase activity was measured by the estimation of glucose formed by the Tris glucose oxidase peroxidase method of Dahlquist (1964). Sucrase was assayed by a similar method but using sucrose instead of trehalose. Protein was estimated by the method of Lowry et al. (1951), using bovine serum albumin as standard. The modified method of Wang and Smith (1975) was used for samples containing Triton X-100. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyse 1 μmol of substrate per min at 37°C.

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*To whom all correspondence should be addressed.

Abbreviations used: PAGE, Polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; PCMB

Coomassie brilliant blue. PAGE in the presence of sodium dodecyl sulphate (SDS) was performed according to the method of Weber and Osborn (1969).

Heat inactivation

The crude brush border membrane was diluted with buffer, to a final protein concentration of 3 mg/ml and kept in a constant temperature bath maintained at 55 °C. Aliquots were withdrawn every 20 min and assayed for trehalase activity.

Purification of the enzyme

A summary of the purification procedure is given in table 1. Adult animals of either sex were used. The small intestines were washed with 1·15% (w/v) KCl, slit open longitudinally, and the mucosa scraped with a blunt knife. The scrapings were homogenised in 0·01 M potassium phosphate buffer, pH 7, in a Waring blender for 30 s in the cold. The homogenate was centrifuged at 13,000 g for 20 min in a Sorvall RC-5B refrigerated centrifuge. The pellet was suspended in half volume of 0·01 M potassium phosphate buffer, pH 7, and homogenised using a teflon pestle. The crude membrane fraction thus obtained was diluted 1:1 with the same buffer as above and treated with 1% (w/v) Triton X-100 at 37°C for 60 min, with occasional stirring. At the end of this time, the suspension was centrifuged at 38,000 g for 4 h. The supernatant which contained over 90% of the trehalase and sucrase activities was raised to 30% saturation with ammonium sulphate, and centrifuged at 13,000 g for 30 min. The precipitate which contained no trehalase activity was discarded. To the supernatant, Triton X-100 was added to a final concentration of 1% and the solution dialysed against 1 mM sodium phosphate buffer to remove the salt. The dialysed supernatant was applied to a column of DE-52 (4×1 cm, bed volume 10 ml) equilibrated with 10 mM sodium phosphate buffer, pH 5·6, containing 1% Triton X-100. Trehalase was located in the flow through and washings. The fractions containing trehalase activity were pooled and dialysed against 1 mM sodium phosphate buffer, pH 6·8, and applied to a column of hydroxyapatite ($1·6 \times 1$ cm, bed volume 5 ml) equilibrated with 1 mM sodium phosphate buffer, pH 6·8 containing

Table 1. Purification of trehalase from monkey small intestine.

Fraction	Activity (units/ml)	Total units	Protein (mg/ml)	Total protein (mg)	Specific activity	Recovery (%)
Pellet	2·5	125	32	1600	0·078	100
Triton X-100 supernatant	1·33	120	4·2	378	0·316	96
30% Ammonium sulphate supernatant	1·00	120	1·5	180	0·666	96
DE-52 flow through	0·75	90	0·46	54·6	1·64*	72
Hydroxyapatite I	2·2	66	0·47	14·07	4·7*	53
Hydroxyapatite II	2·4	24	0·22	2·28	10·0*	10

acia GM-1 gradient maker. Trehalase eluted as a single sharp symmetrical
The fractions showing trehalase activity were pooled and dialysed against
sodium phosphate buffer pH 6·8 and applied to a second column of hydroxy-
(bed volume 1 ml) equilibrated with 1 mM sodium phosphate buffer pH 6·8.
lumn was washed with the equilibrating buffer and eluted with a linear
it from 20–100 mM sodium phosphate buffer pH 6·8. The fractions showing
se activity were pooled and used for subsequent experiments.

rified enzyme was free of maltase and sucrase activities. The enzyme was
1 at different pH values from pH 3–7 using citrate phosphate buffer in a
assay mixture. Trehalase activity exhibited a sharp peak at pH 5·8. The
showed a K_m of 17·4 mM for trehalose and a V_{max} value of 1·33 units at
with sodium phosphate buffer. The variation of K_m with pH was studied
citrate phosphate buffer at different pH values. The plot of pK_m versus pH
o indicate the involvement of two groups with pK values 4·5 and 6 (figure 1).
e inhibits the enzyme competitively with a K_i value of 22·6 mM (Dixon and
1979) (figure 2). Tris was found to be a competitive inhibitor of the enzyme
K_i value of 19·5 mM (figure 3). p-Chloromercuribenzoate (PCMB) inhibits
se activity. About 50% of the activity is inhibited by 3·33 mM PCMB and
py 10 mM PCMB. Mercuric chloride (0·4 mM) totally abolishes enzyme
. At 55°C, about 70% of the enzyme activity was lost at the end of 1 h. The
1 activity when maintained at 60°C, was totally denatured at the end of
The crude membrane fraction as well as the partially purified fraction
d identically.
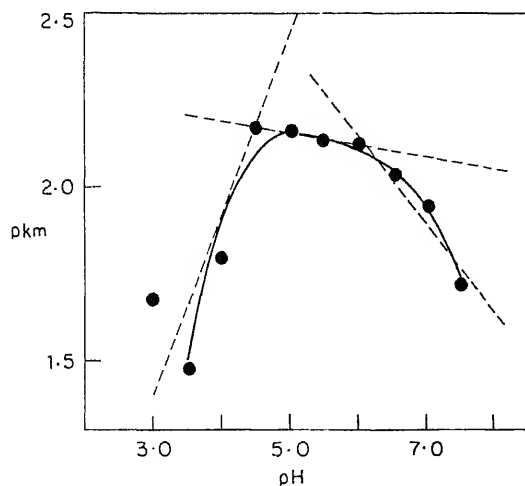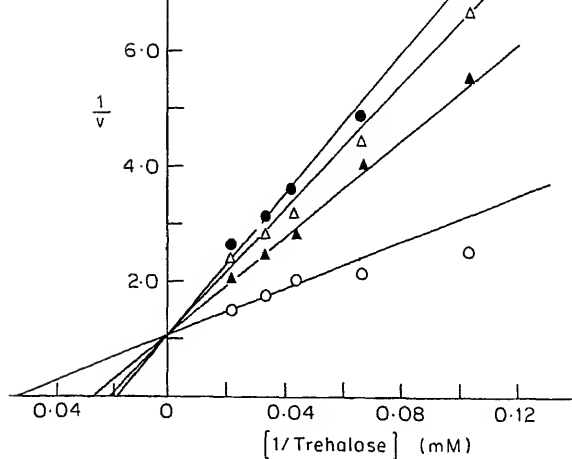
Figure 2. Inhibition of trehalase activity by sucrose. Line-weaver-Burk plots of velocities in the absence of sucrose (O) and in the presence of 20 mM sucrose (▲), 40 mM sucrose (△) and 60 mM sucrose (●).
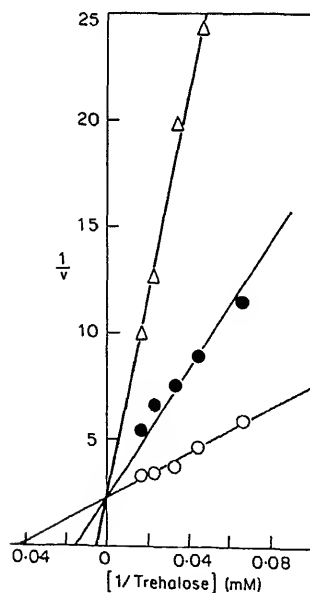
Figure 3. Inhibition of trehalase activity by Tris. Lineweaver-Burk plots of velocities in the absence of Tris (O) and in the presence of 50 mM Tris (●) and 100 mM Tris (△).

Molecular weight of the enzyme

The purified enzyme was apparently homogeneous since it moved as a single band

band, corresponding to a M_r of 28,000 were seen. However, upon reduction β-mercaptoethanol, only one band was seen, with a M_r of 28,000; the heavier of 138,000 was not seen after reduction (figure 4).
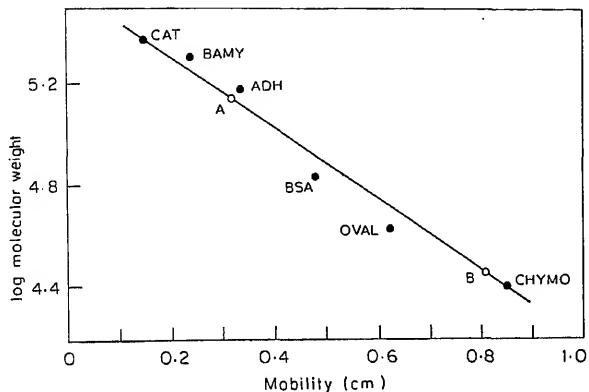
Figure 4. Estimation of the M_r of trehalase by SDS-PAGE. The standard markers used were catalase (243,000), alcohol dehydrogenase (150,000), β-amylase (200,000), bovine serum albumin (66,000), ovalbumin (42,000), and chymotrypsinogen (25,000). **A.** SDS treated. **B.** SDS/β-mercaptoethanol treated.

ssion

lase has been purified from the brush border membrane of the monkey small ne by a relatively simple procedure after solubilisation with Triton X-100 with erall recovery of 19%. Subsequent to solubilisation, it was necessary to ain 1% Triton X-100 throughout the purification process, in the absence of the ent, trehalase was rapidly inactivated. Sasajima et al. (1975) have used butanol tion to remove Triton X-100. We found that monkey trehalase activity was yed totally during extraction of Triton supernatant or crude membranes with nol. Precipitation with 80% ammonium sulphate also results in denaturation alase activity. Hence, in our procedure, some of the proteins were removed by itation with 30% ammonium sulphate and the supernatant containing soluble ase activity was passed through a DE-52 column in the presence of 1% Triton . The results seem to indicate that the trehalase of the monkey small intestine is ly hydrophobic protein. Though trehalase has been purified from different s, its molecular properties like size and quaternary structure are not well stood. Recently, Yokota et al. (1986) have isolated an amphiphilic trehalase rabbit small intestine by solubilisation with Triton X-100 in the presence of . The presence of EDTA inhibits endogenous proteinases and hence the dure results in trehalase carrying the hydrophobic tail which appears to be less 5,000 in M_r. However, the hydrophobic anchor peptide corresponding to ase has not so far been isolated from any source.

binding of higher amounts of Triton X-100. The enzyme appears to give products of M_r 28,000 upon reduction. Nakano *et al.* (1977) reported the presence of a polypeptide of M_r 30,000 in the rat small intestine. Recently, Yokota *et al.* (1986) observed that though their preparation of trehalase behaved as a single protein under nondenaturing conditions, it showed a protein band corresponding to a M_r of 30,000 on SDS gel electrophoresis. This was thought to be a proteolytic product but still associated with the protein of M_r 75,000. Such anomalies have long been known during SDS gel electrophoresis (Dreyer *et al.*, 1972; Wallach and Winzler, 1974). Hence it appears reasonable to conclude that the trehalase from the monkey intestine is a monomeric protein of M_r 138,000. Further studies are in progress to resolve the quarternary structure.

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Isolation, purification and partial characterisation of prealbumin from cerebrospinal fluid[§]

M. V. BIMANPALLI and P. S. GHASWALA*†

Department of Biochemistry, Grant Medical College, Bombay 400 008, India
*Present address: Sir Hurkisondas Nurrotumdas Medical Research Society, Raja Rammohan Roy Road, Bombay 400 004, India

Abstract. Prealbumin from human cerebrospinal fluid was purified using a combination of ammonium sulphate precipitation, phenol precipitation, polyacrylamide disc gel electrophoresis and gel filtration on Sephadex G-100. The homogeneity of the purified protein was established by polyacrylamide gel electrophoresis and immunoelectrophoresis. On the basis of its molecular weight (55,000), amino acid composition, electrophoretic mobility and immunological cross-reactivity, the prealbumin from cerebrospinal fluid showed complete identity with serum prealbumin. The cerebrospinal fluid prealbumin levels in various neurological disorders may have a diagnostic significance.

Keywords. Prealbumin; protein purification; gel electrophoresis; gel filtration; immunoelectrophoresis; amino acid analysis. ·

Introduction

Prealbumin is a plasma protein with electrophoretic mobility greater than that of serum prealbumin. A lot of information on the structure and physicochemical properties of human serum prealbumin is available (Goodman, 1974; Kanda *et al.*, 1974). Prealbumin plays an important role in the plasma transport of vitamin A and thyroid hormones. Navab *et al.* (1977) suggested that the role of prealbumin in vitamin A transport in rats appears to be comparable to its role in humans. Although prealbumin appears to be the major transport protein in rats (Davis *et al.*, 1970), it plays a secondary role in humans since thyroid binding globulin is the major transport protein for thyroid hormones (Smith *et al.*, 1983).

The literature available on the nature and molecular form of prealbumin from human cerebrospinal fluid (CSF) is contradictory. Schultz and Heremans (1966) reported that the prealbumin fraction of CSF is quantitatively very important. The protein has been identified and shown to be similar to prealbumin from serum (Schultz *et al.*, 1956). On the other hand, Laurell (1972) reported partial immunological identity between serum and CSF prealbumins while Stibler (1978) reported that CSF prealbumin has a lower isoelectric pH than the serum protein. A slightly higher electrophoretic mobility of CSF prealbumin compared to that of serum prealbumin was noted by Jeppsson *et al.* (1979). It was postulated that the bulk of CSF proteins is derived from the plasma by a process of molecular sieving. This concept has been verified more directly with the aid of $[^{131}I]$-labelled albumin and

intravenous route. Despite the abundance of information on fractional studies [of] spinal fluid in neurological disorders (Hordynsky, 1972; Kamath *et al.*, 1974; Phad[e] *et al.*, 1975; Sundervalli *et al.*, 1979), only a few useful clinical correlations have be[en] drawn for CSF prealbumin and a great deal still needs to be appreciated. Apart fr[om] the absence of CSF prealbumin in lumbar fluid in cases of complete block in [the] spinal cord (Hill *et al.*, 1959) and tubercular meningitis (Sridhara Ramarao, 19[..]) little is known about its involvement in pathology.

In order to evaluate the diagnostic value of CSF prealbumin, a detailed a[nd] systematic study was initially undertaken to clarify the relationship between ser[um] and CSF prealbumin and thereafter to accurately estimate CSF prealbumin levels [in] various neurological disorders using simple routine methods such as single rad[ial] immunodiffusion.

Materials and methods

Materials

The materials used in the study were procured from the following sources: acrylam[ide] from BDH Chemicals, Poole, UK; Blue dextran and molecular weight M_r mark[ers] such as bovine serum albumin (BSA), egg albumin, myoglobin and cytochrom[e] were from Sigma Chemical Co., St. Louis, Missouri, USA; liver albumin fr[om] Nutritional Biochemical Corp., Ohio, USA; Noble agar and Freund's comp[lete] adjuvant from Difco Laboratories, Detroit, Michigan, USA; β-mercaptoetha[nol] from E. Merck, Darmstadt, West Germany; Sephadex G-100 from Pharmacia F[ine] Chemicals, Piscataway, New Jersey, USA. All other chemicals used were of analyt[ical] grade unless stated otherwise.

Purification

Samples of CSF which were referred for routine investigations were collected [by] lumbar puncture without any blood contamination and stored at $-20°C$ until [..] Prealbumin was purified using 4 simple steps *viz.* ammonium sulphate saturat[ion,] phenol precipitation, polyacrylamide disc gel electrophoresis (PAGE) and [gel] filtration on a column of Sephadex G-100 as shown in figure 1. Protein [was] determined using the method of Lowry *et al.* (1951).

The purity of prealbumin was double-checked by PAGE (Davis and Ornst[ein,] 1964) and immunoelectrophoresis (Kelkar and Khare, 1984) using rabbit anti-hu[man] prealbumin, and anti-prealbumin from Behringwerke-Hoechst India Lim[ited,] Bombay.

Preparation of antiserum

A rabbit weighing approximately 2 kg was bled before immunisation and the se[rum]

Stored overnight at 4°C
Centrifuged at 10,000 g for 30 min

Supernatant
(discard)

Precipitate + 0·18 M saline

Dialysed and concentrated
Centrifuged at 5,000 g for 15 min

Supernatant
+ 5% (w/v) phenol — Step II

Precipitate
(discard)

Centrifuged at 10,000 g for 15 min

Supernatant

Precipitate
(discard)

Dialysed and concentrated

PAGE — Step III

Prealbumin fraction eluted at 4°C in 0·18 M saline

Gel filtration on
Sephadex G – 100 — Step IV

Purity check

PAGE   Immunoelectrophoresis

Homogeneous CSF Prealbumin

Figure 1. Procedure for purification of human CSF prealbumin.

given intra-cutaneous and intra muscular injections of the antigen at multiple sites as described by Muto and Goodman (1969). Four weeks later a booster injection was given and at one week thereafter, blood was collected from the ear veins. Double immunodiffusion was carried out by the method of Ouchterlony (1962) and Ouchterlony and Nilsson (1973). Two weeks later one more booster injection was given to increase the titre of the antiserum. Antiserum thus obtained was stored at $-20°C$ with 0·2% sodium azide as preservative and thereafter used for quantification and immunochemical characterisation of prealbumin in CSF.

M_r determination

The M_r of prealbumin from CSF was estimated by sodium dodecyl sulphate (SDS)-PAGE (Weber and Osborn, 1969) and by gel filtration on a standardised column of

the mixture in a 90°C water bath for 5 min. The sample was then mixed with 10 μl of bromophenol blue and 8 M sucrose solution and applied on gels for SDS-PAGE.

Amino acid analysis

Purified prealbumin from CSF (0·5 mg) was hydrolysed in 1 ml of 6 N HCl in evacuated sealed tubes for 24 and 48 h at 110°C. HCl was removed by evaporation *in vacuo*. Amino acid analysis was performed by the method of Spackman *et al.* (1958) on a Beckman 119 C L Amino Acid Analyser. Quantitative determination of half-cystine and methionine were carried out by partial oxidation. The tryptophan content of prealbumin was estimated by the method of Goodwin and Morton (1946).

Ultraviolet absorption data: The ultraviolet (UV) absorption spectrum was obtained by using a sample of purified prealbumin (0·5 mg/ml) and an automatic recording spectrophotometer (Perkin-Elmer Lambda 3B UV/Vis).

Results and discussion

The present study deals with the isolation, purification and partial characterisation of prealbumin from human CSF.

Purification

Prealbumin was purified from CSF using 4 simple steps, *viz*, ammonium sulphate saturation, phenol precipitation, PAGE and gel filtration on Sephadex G-100. The purification achieved is summarized in table 1. Ammonium sulphate saturation and phenol precipitation resulted in the elimination of undesired proteins (figure 2) and 27·7-fold purification. PAGE resulted in further purification (31·87-fold) and the overall recovery after Sephadex G-100 chromatography (figure 3) was 56% and 5·4 mg of pure prealbumin was obtained (table 1). The final preparation gave upon PAGE only one protein band with anodal mobility greater than that of CSF albumin (figure 2). A single precipitin band corresponding to prealbumin was

Table 1. Purification of prealbumin from human CSF.

	Total protein (mg)	Total prealbumin (mg)	Overall recovery (%)	μg of prealbumin per mg of protein	Purification factor relative to whole CSF
CSF	316·00	9·60	100	30·38	1·00
90% (NH₄)₂SO₄ saturation	300·00	9·40	98	31·33	1·03
Phenol precipitation	10·60	8·60	89	811·20	27·70
PAGE	6·20	6·00	78	967·90	31·87
Sephadex G-100 chromatography	5·44	5·40	56	993·00	32·69
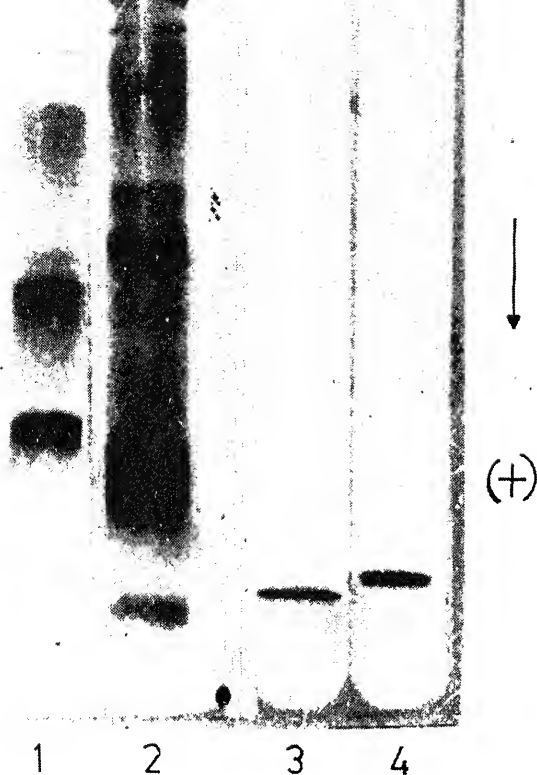
Figure 2. Analytical PAGE of CSF proteins. (1), Whole CSF; (2), after $(NH_4)_2 SO_4$ precipitation; (3), after phenol precipitation; (4) purified prealbumin.
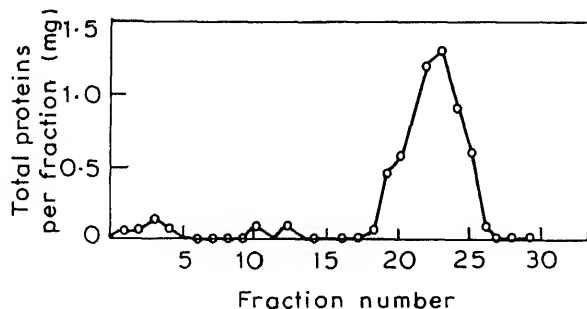
Figure 3. Column chromatography of CSF prealbumin on Sephadex G-100. The material from PAGE was applied to a column of 1·6 cm (i.d.) × 82 cm. Elution was carried out with 0·02 M phosphate buffer containing 0·2 M NaCl at a flow rate of 11 ml/h. 2 ml fractions were collected after collecting a void volume of 40 ml. Protein in each fraction was assayed by the method of Lowry *et al.* (1951).

Figure 4. Immunoelectrophoresis. (1, 1a), Purified prealbumin; (2), CSF after $(NH_4)_2SO_4$ precipitation against poly-specific antiserum enriched in anti-prealbumin; (2a), CSF after $(NH_4)_2SO_4$ precipitation against prealbumin-specific antiserum.

Immunologic studies

Evidence of partial immunological identity between CSF and serum prealbumins was given by Laurell (1972). In the present study, a single precipitin line was obtained in double immunodiffusion between rabbit antiserum to human CSF prealbumin and purified human CSF prealbumin (figure 5). A single precipitin line was also obtained between the antiserum and whole CSF. No precipitin reaction was observed between the antiserum and human serum albumin. However a single preci-
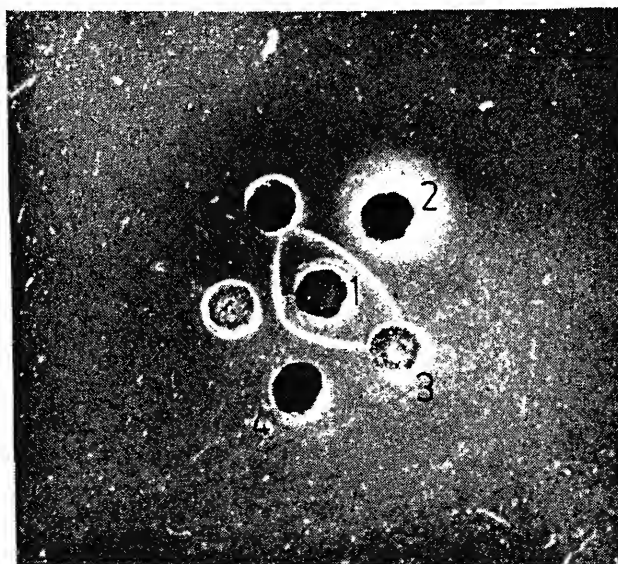
Figure 5. Double immunodiffusion. (1), Antiserum to human CSF prealbumin; (2), whole CSF; (3), human serum albumin; (4) purified human CSF prealbumin; (5), human serum prealbumin.

line was obtained between antiserum and serum prealbumin. This line was
nuous with that between antiserum and CSF prealbumin and there was no
nce of spur formation (figure 5) indicating that CSF and serum prealbumins are
nologically identical.

ous workers have reported different M_r for human serum prealbumin (Schultz
1956; Raz and Goodman, 1969; Kanda *et al.*, 1974). A M_r of 54,980 is in close
ment with an estimate based on a crystallographic method using unit cell data
e *et al.*, 1974) and the M_r calculated from the complete amino acid sequence
ted by Kanda *et al.* (1974). In the present study, the M_r of human CSF
bumin was estimated to be 55,000 from SDS-PAGE and gel filtration data
es 6 and 7). Thus the M_r of human CSF prealbumin is almost the same as that
man serum prealbumin.
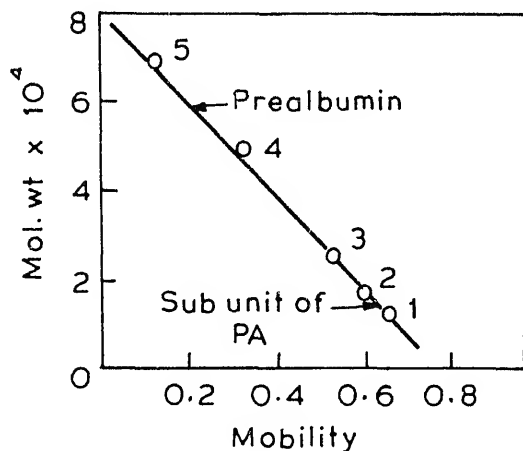
Figure 6. M_r determination by SDS-PAGE. The standard proteins used were (1),
Cytochrome *c*; (2), myoglobin; (3), α-chymotrypsin; (4), egg albumin; (5), liver albumin.

nits

PAGE under reducing and denaturing conditions resulted in the loss of the
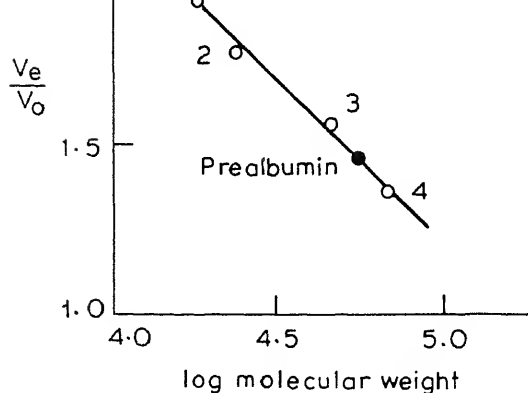
Figure 7. M_r determination by gel filtration. A portion of the purified prealbu[min]
preparation was chromatographed on a column of Sephadex G-100, 1.6×85 cm in
using 0·02 M phosphate buffer (pH 7·6) containing 0·2 M NaCl with a flow rate of 11 m
The column was standardised by using small samples (2–4 mg) of proteins of known M
small amount of blue dextran polymer (M_r 2×10^6) was added to the protein sample be
chromatography in order to determine void volume (V_o). The standard proteins were
myoglobin, (2), α-chymotrypsin, (3), egg-albumin, and (4) BSA.

UV spectrum

The UV absorption spectrum of purified CSF prealbumin shows maximum absorp[tion]
at 278 nm (figure 8). The extinction coefficient ($E_{1cm}^{1\%}$) was found to be 14·1 at 280
when measured with a solution containing 0·5 mg/ml of the protein.

Plasma prealbumin[c]
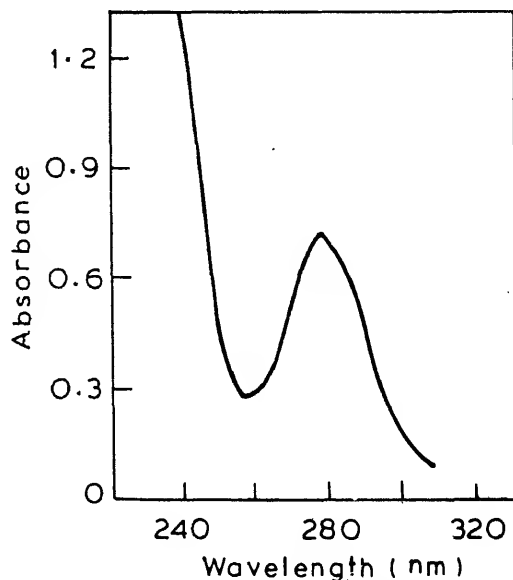
Amino acid	Hydrolysis 24 h (μmol × 10²)	48 h (μmol × 10²)	Corrected[a] distribution of μmol (%)	μmol of each amino acid relative to histidine[b]	Estimated no. of residues per molecule	No. of residues per subunit	Observed CH_3SO_3H hydrolysis (μmol × 10²)	No. of residues per subunit
Lysine	12·64	13·28	6·40	32·00	32	8	8·30	8
Histidine	06·51	06·32	3·20	16·00	16	4	04·00	4
Arginine	06·18	06·26	3·14	15·70	15–16	4	04·20	4
Aspartic acid	13·64	13·06	6·43	32·10	32	8	07·57	8
Threonine	18·38	18·19	9·64	48·20	48	12	11·65	12
Serine	16·86	16·18	8·89	44·40	44	11	10·65	11
Glutamic acid	20·10	18·82	9·60	48·00	48	12	11·60	12
Proline	13·46	13·32	6·42	32·10	32	8	08·85	8
Glycine	15·86	16·48	8·08	40·40	40	10	10·05	10
Alanine	20·30	19·74	9·72	48·60	48–49	12	11·90	12
Half cystine	01·32	—	0·66	03·30	3–4	1	—	1
Valine	18·62	18·92	9·62	48·10	48	12	10·71	12
Methionine	01·24	—	0·65	03·20	3–4	1	00·90	1
Isoleucine	08·73	08·46	4·04	20·20	20	5	5·03	5
Leucine	11·24	11·90	5·53	27·65	28	7	742	7
Tyrosine	07·86	08·02	3·96	19·80	20	5	5·23	5
Phenylalanine	07·89	08·22	4·02	20·10	20	5	5·48	5
Tryptophan	—	—	—	—	8	2	1·74	2
Residues	200·93	191·17	100·00	—	505–509	127	125–129	127

[a]The corrected percentage distribution lists the average of two values for amino acids (24 and 48 h) except: (i) threonine and serine for which the observed values were extrapolated back to zero time and these values were used, (ii) half cystine which was measured separately and (iii) while tryptophan which was measured with a spectrophotometer and by using the molar ratios of tryptophan and tyrosine the final values were adjusted such that their sum was equal to 100%.

[b]Relative to histidine which was assigned the value of 16.

[c]Kanda et al. (1974).

CSF prealbumin was analysed after hydrolysis with 6 N HCl and the results are presented in table 2. The results are in good agreement with the known composition of plasma prealbumin (Kanda *et al.*, 1974). Relatively high proportions of aromatic amino acids such as tyrosine and tryptophan (Goodwin and Morton, 1946) were observed in CSF prealbumin as is the case with plasma prealbumin.

Prealbumin from CSF closely resembles serum prealbumin with regard to many of its characteristics. The extinction coefficient of CSF prealbumin is almost the same as that of serum prealbumin (Raz and Goodman, 1969). The presence of relatively high proportions of tyrosine and tryptophan (table 2) in these proteins is responsible for their fairly high extinction coefficients. The UV absorption spectrum of CSF prealbumin is also almost identical with that of prealbumin (Peterson, 1971). The similarities observed between CSF and serum prealbumin suggest that the two proteins are identical.

Prealbumin has been reported to have a lower isoelectric pH (Stibler, 1978) and a higher electrophoretic mobility (Jeppsson *et al.*, 1979) than serum prealbumin. In the present study it was shown that purified serum prealbumin has the same electrophoretic mobility as that of CSF prealbumin (figure 9). Furthermore, the similarity of their equivalence points and lack of spur formation in the continuous precipitin line
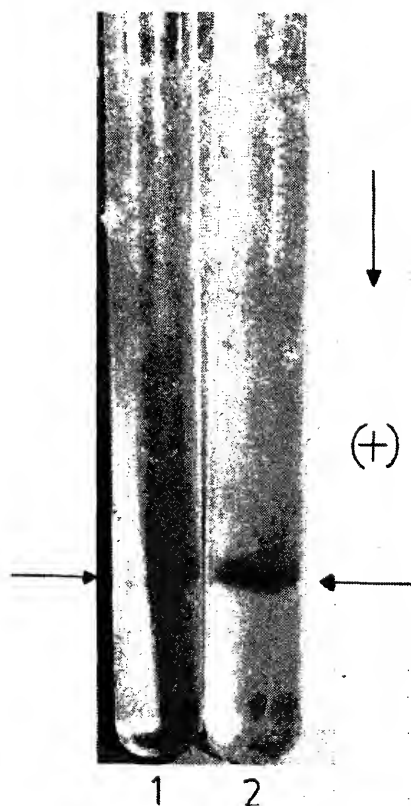
the prealbumins from CSF and serum are identical. The differences noticed by
...ll (1972) in the immunological equivalents of prealbumins from serum and CSF
...e due to retinol binding protein and other proteins in serum which may have
...atrix effect' on the shape of the rockets in electroimmunoassay of prealbumin.
...nclusion, we may mention that serum and CSF prealbumin are identical
...ns.

...th the use of antisera to purified prealbumin, we are now in a position to
...ately measure changes in prealbumin levels in abnormal cerebrospinal fluids.
...ninary results indicate that prealbumin levels may play a significant role in
...logical disorders and may be of use in clinical diagnosis.

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Structural similarities among the high molecular weight protein fractions of oilseeds

V. PRAKASH* and M. S. NARASINGA RAO

Central Food Technological Research Institute, Mysore 570 013, India

Abstract. Data on the physico-chemical properties of proteins from soybean, groundnut, sesame seed, sunflower seed, safflower seed, mustard seed, rapeseed and cotton seed are fairly extensive. An examination of the available data on high molecular weight proteins suggests that there are similarities in many of their properties. In this report the similarity in amino acid composition, size and shape, molecular weight, secondary structure, subunit composition, association-dissociation at high and low pH, stability towards denaturants, hydrolysis by enzymes and quaternary structure of the high molecular weight proteins is discussed. Based on these similarities a model has been proposed for the association-dissociation, denaturation and reassociation behaviour of the high molecular weight proteins of oilseeds.

Keywords. Structural similarity; oilseed proteins; high molecular weight proteins; oilseeds; model for seed protein; association-dissociation; denaturation.

Introduction

Oilseed proteins have in recent years attracted the attention of scientists in view of their importance in the plant system and their unique properties. Of the two major classes of plant proteins, namely, functional proteins and reserve proteins, the latter have been studied in great detail (Prakash and Narasinga Rao, 1986). Pernollet and Mosse (1983) have described these proteins as tropic secretory proteins able to associate and exhibit relative multiplicity and polymorphism. Their deposition in the seed is shown to be close to phosphate reserves in the cell. This article highlights the similarities in the composition and properties of these proteins. An attempt has been made to correlate structure with function in the seed, based on their solution properties.

The literature on the physico-chemical properties of some of the oilseed proteins such as those from soybean, groundnut, sesame seed, sunflower seed etc. is fairly extensive (Prakash and Narasinga Rao, 1986). In general the proteins of oilseeds can be categorised into two groups, namely, the high molecular weight (M_r) protein fraction and the low M_r protein fraction. Since extensive data is available at present on the high M_r proteins, the discussion is confined to this class.

If one carefully examines the reported data on protein fractions of various oilseeds a great similarity is apparent in (i) the number of fractions and (ii) their sedimentation coefficients. Table 1 summarises available data. All oilseeds consist predominantly of 4 protein fractions. These can be designated for the purpose of discussion as 2S (low M_r protein fraction), 7S (medium M_r protein fraction, corresponds to vicillin of legumes), 11S (high M_r protein fraction, corresponds to legumin of legumes) and 15–18S ('polymer' resulting from possible aggregation of 2S, 7S, or 11S or may be

Source	Protein fraction			Polymer
	Low M_r	Medium M_r	High M_r	
Soybean	2	7	11	15
Groundnut	2	7	11	18
Sesame seed	2	7	11	15
Sunflower seed	2	7	12	16
Mustard seed	2	7	12	—
Rapeseed	2	7	12	—
Cottonseed	2	7	11	18
Safflower seed	2	7	12	17

Data from Prakash and Narasinga Rao (1986).

inherently present in the seed). It is observed that substances such as try inhibitors, hemagglutinins, polyphenols, glucosinolates, colour and bitter princi which are inherent constituents of some of the oilseeds, are generally associated n with the 2S or 7S fraction. However one cannot rule out such association being artifact of isolation procedures.

The high M_r protein fraction (10–12S) is the major fraction in groundnut (Prak and Narasinga Rao, 1986), sesame seed (Prakash and Nandi, 1978), sunflower (Schwenke et al., 1974) and safflower seed (Latha and Prakash, 1986). On the c hand, in soybean, mustard seed, rapeseed and cottonseed it is present to the exten only 20–30% (Prakash and Narasinga Rao, 1986). However, in these seeds als forms a significant proportion of the total proteins.

In this report the similarity in amino acid composition, size and shape, secondary structure, subunit composition, association-dissociation at high and pH, stability towards denaturants, hydrolysis by enzymes and quaternary struc of the high M_r proteins from various oilseeds will be discussed.

Amino acid composition

The amino acid composition of the high M_r proteins from 7 different seeds are g in table 2. All the high M_r proteins are rich in acidic amino acids, especially glut acid, and aromatic amino acids, and are low in lysine. Mosse (1973) and Pern and Mosse (1983) have reviewed the intraspecific variation in amino acid position of legume seeds. In this review we have analyzed the amino acid da terms of hydrophobicity and other related indices.

Hydrophobicity and related indices

The fundamental structural parameters of proteins which depend only on amino composition have been shown to be Waugh's NPS or frequency of non-polar chains (Waugh, 1956; Bigelow, 1967), Fischer's P or the ratio of the volume occu by polar residues to that occupied by non-polar residues (Bigelow, 1967; F

no acid	Glyci-nin[a]	Ara-chin[b]	α-glob-ulin[c]	Helia-nthin[d]	Bras-sin[e] (M)	Bras-sin[f] (R)	Carmin[g]	High M_r gliadin from wheat[h]	Rice globulin[i]
artic acid	106	111	84	107	53	83	110	17	68
onine	44	19	41	36	28	38	27	22	29
ne	74	57	59	51	30	56	62	60	63
amic acid	169	171	155	197	134	162	181	324	91
ine	50	40	21	52	ND	43	46	130	57
cine	64	59	90	85	75	81	92	42	103
ine	47	40	71	69	34	54	67	23	128
ne	43	34	46	63	48	39	38	35	63
hionine	9	1	20	19	22	14	10	11	18
cystine	7	8	7	11	ND	ND	10	11	0
eucine	45	25	32	49	52	32	23	29	27
cine	56	60	63	68	9	66	57	64	58
osine	24	28	24	20	15	19	32	15	31
nylalanine	34	31	34	48	23	33	21	35	22
ne	33	26	16	19	17	23	17	9	20
idine	17	17	20	23	13	21	17	12	12
nine	45	92	91	66	ND	39	64	22	70
otophan	7	11	11	10	9	9	11	ND	7

amino acid compositions of gliadin from wheat and rice globulin are given for comparison.

mbers are residues per molecule of protein. ND, Not determined.

arts and Vaintraub (1967); [b]Tombs (1965); [c]Prakash and Nandi (1978); [d]Schwenke et al. (1974); uraj Rao and Narasinga Rao, (1981); [f]Gill and Tung (1978); [g]Latha and Prakash (1986); [h]Bietz and (1973); [i]Tecson et al. (1971).

ironment to an aqueous environment. In table 3 the average hydrophobicity, S and P values of high M_r proteins are listed. Also included in table 3 are data on

Table 3. Calculated values of hydrophobicity and related parameters of the high M_r proteins of vrious oilseeds.

Protein	HQ[a]	NPS[b]	P[c]
Ovalbumin	1110	0·34	0·92
Fibroin (silk)	440	0·02	0·07
Glycinin	782	0·30	1·28
Arachin	860	0·29	1·73
α-Globulin	872	0·26	1·36
Helianthin	832	0·26	1·25
Brassin (M)	962	0·31	1·03
Brassin (R)	900	0·30	1·00
Gossypin	804	0·24	1·00
Carmin	824	0·26	1·69
Poppyverin	878	0·28	1·49
Linin	881	0·27	1·22
Average ± SD	860 ± 50	0·28 ± 0·02	1·31 ± 0·25

Values for ovalbumin and fibroin are included for comparison.

[a]average hydrophobicity.

[b]Frequency of non-polar side chains.

values were calculated by the methods described in the papers cited above.

Two interesting observations emerge from this analysis. Firstly, the values of th[e] parameters are nearly the same for all the high M_r proteins except for gossy[pin] whose values of average hydrophobicity and NPS are all lower than the aver[age] value. Except for the value of P which ranges from 1 to 1·73 the values of the ot[her] parameters are remarkably close to each other indicating a general trend. This m[ay] be related to the fact that these proteins have a low proportion of α-helix and a h[igh] content of β- and aperiodic structure. Further, the values for the high M_r proteins [do] not fit any portion of the Bigelow plot of average hydrophobicity vs M_r (Bigel[ow,] 1967). Similarly the NPS values of high M_r proteins are higher than those repo[rted] for other proteins (Waugh, 1956; Bigelow, 1967).

Secondary structure

The circular dichroic spectra of the high M_r proteins are generally characterised [by a] minimum around 208–212 nm with a shoulder around 224–228 nm. This a[lso] suggests that the high M_r proteins have a low proportion of α-helix and are rich i[n β-] and aperiodic structure. Table 4 summarises the secondary structure data for [the] high M_r proteins. They all have less than 10% α-helix and 20–30% β-structure, [the] rest being aperiodic structure. However, attempts have not been made to calcu[late] from the circular dichroism (CD) data the number of β-turns. Blake and Joh[nson] (1984) have classified proteins into 5 classes based on secondary structure. These [are] defined in terms of the proportions and arrangement of α-helix and β-sheet. [The] various classes are (i) all-α proteins, (ii) all-β-proteins, (iii) $\alpha + \beta$ proteins, (iv) [...]

Table 4. Intrinsic viscosity, secondary structure, M_r, number of subunits and carbohydrate cont[ent of] the high M_r proteins from various oilseeds.

Protein	Viscosity, η (ml/g)	Secondary structure (%)			$M_r \times 10^{-5}$	No. of subunits	Ca[rbo-] hyd[rate] ([%)]
		α-Helix	β-Structure	Aperiodic			
Glycinin[a]	4·9	5	20	75	3·0–3·5	6	–
Arachin[a]	4·7	5	20	75	3·0–3·3	6	[0]
α-Globulin[a, b]	3·0	5	25	70	2·3–2·7	6	[0]
Helianthin[c]	3·6	2	28	70	3·0–3·5	6	[0]
Brassin (M)[d]	3·6	9	28	63	2·3–2·4	6(8)	1
Brassin (R)[e]	3·7	9	28	63	2·9–3·0	6	1
Gossypin[f]	4·0	5	20	75	2·2–2·5	6(5)	[0]
Carmin[g]	3·7	3	15	82	2·4–2·9	6	1
Poppyverin[h]	3·5	5	20	75	2·0–2·3	6	1
Linin[i]	3·1	3	17	80	2·5–3·0	6	[0]
Ribonuclease[j, k]	3·3	40	13	24	—	—	–
Collagen[j]	1150	—	—	—	—	—	–
Elastase[k]	—	7	52	26	—	—	–

Data for ribonuclease, collagen and elastase included for comparison.

[a]Prakash and Narasinga Rao (1986); [b]Prakash (1985); [c]Schwenke et al. (1974); [d]Gururaj Ra[o and] Narasinga Rao (1981); [e]Gill and Tung (1978); [f]Reddy and Narasinga Rao (1984) and Li et al. [...]; [g]Latha and Prakash (1986); [h]Srinivas (1984); [i]Madhusudan (1984); [j]Tanford (1961); [k]Prasad ph [...]

ins and (v) 'coil proteins'. The high M_r proteins of oilseeds do not seem to fit
ny of these classes since they are rich in β-sheet and aperiodic or coil structure.
h class termed β + coil proteins, may be appropriate to describe these proteins.

sic viscosity

ntrinsic viscosities of the high M_r proteins from various oilseeds are given in
4. They all have an intrinsic viscosity value between 3 and 5 ml/g. Based on
rd's criterion for globular proteins all the high M_r proteins appear to be
lar in shape. For comparison, the values for ribonuclease, which is a globular
in, and collagen, a highly asymmetric protein, are also shown in table 4. These
also indicate a generality among the high M_r proteins.

uld be pointed out that different workers have used different techniques such as
dimentation-diffusion method, sodium dodecyl sulphate (SDS)-polyacrylamide
lectrophoresis (PAGE), aproach-to-equilibrium method in the analytical
entrifuge etc. to determine the M_r's of the high M_r proteins. The values
ned by the different methods are not strictly comparable. However, they are in
nge 2×10^5 to 3.5×10^5. The estimation of M_r by a thermodynamically sound
od such as by sedimentation equilibrium in a single solvent is clearly indicated.
in a few cases has this technique been used (Prakash, 1985; Latha and Prakash,

escence

luorescence emission maximum of the high M_r proteins is around 320–330 nm
ash and Narasinga Rao, 1984). The fluorescence emission spectrum suggests
n these proteins the tryptophan residues are embedded in the interior of the
ule. This is compatible with a highly compact globular shape for the protein.
ever, all the proteins contain a fair amount of tyrosine. There are 7–11
ophans and 15–32 tyrosines per molecule of each protein (table 2). But tyrosine
ion is not observed. Shifrin et al. (1971) and Teale (1960) have shown that
ophan fluorescence dominates over tyrosyl fluorescence. The results suggest
here is similarity in the location and microenvironment of non-polar groups in
terior of the molecules of these proteins in spite of the fact that these proteins
s very little α-helical structure. It has been shown that the aromatic amino acids
globulin are in the subunit contact areas stabilizing the quaternary structure
ash, 1985).

olysis

in vitro hydrolysis of the high M_r proteins by proteolytic enzymes such as

of glycoproteins protect them against hydrolysis by proteolytic enzymes. Most of
high M_r proteins are reported to contain carbohydrates (table 4).

Carbohydrate content

Most high M_r proteins have $\sim 1\%$ carbohydrate (table 4). The question that aris
are the high M_r proteins glycoproteins and if so, what role does the carbohyd
play? All these proteins are oligomeric proteins. Possibly the carbohydrate moi
have a role in subunit interactions, folding of the protein molecule into a com
structure and offering resistance to proteolysis (Sharon and Lis, 1981; Sharon, 1

Subunit composition

Since these are large proteins, the energetics of protein assembly and faithful synth
would favour the assembly of subunits to form the native protein. This is
firmed by the oligomeric nature of the proteins (table 4). Most of the high M_r
teins have between 6 and 8 subunits, as determined by SDS-PAGE. The sub
M_r's are in the range 7,000–25,000, indicating a wide range of length of
polypeptide chain. However, these values have been confirmed by other techni
in only a few cases. Pernollet and Mosse (1983) have proposed a general mode
the quaternary structure of legumin invoking a pair of acidic and basic sub
interlinked through S-S bonds and 3 such pairs for a molecule. These are stabi
mostly by non-covalent interactions such as hydrogen bonds and hydrophobic
other weak interactions, and form a 'doughnut' like structure. Two such trimer
sandwiched one above the other such that the acidic and basic subunits a
contact with each other forming a small hollow cylinder (figure 1).

Association-dissociation

All the high M_r proteins exhibit the property of association and dissociation.
process depends on (i) pH, (ii) ionic strength (μ), (iii) protein concentration (C)
(iv) temperature. It seems to depend also on other factors such as the nature o
buffer ions and the presence of other proteins which possibly act as nucle
centres for the association-dissociation process. Based on the available experim
data (Prakash and Narasinga Rao, 1984) a general scheme for the associa
dissociation of high M_r proteins may be proposed as follows.

Association $\xleftrightarrow{\hspace{2cm}}$ Dissociation

μ, pH,	μ, pH,		
temperature	temperature	high or	4
and C	and C	low pH	3
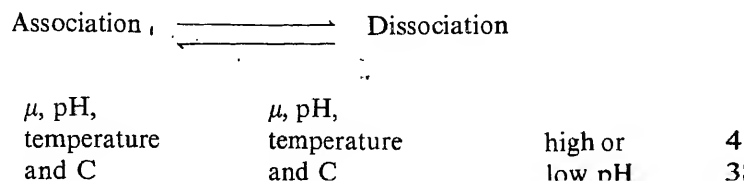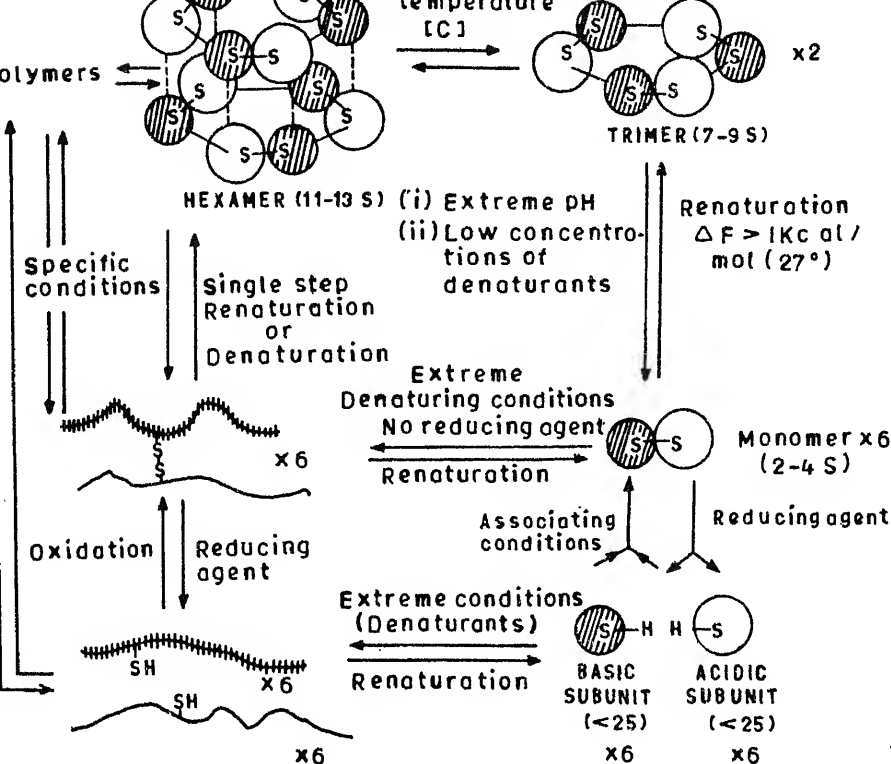
Figure 1. Proposed model for the association-dissociation, denaturation and reassociation of the high M_r protein fraction from various oilseeds. Part of the model is similar to the one proposed by Pernollet and Mosse (1983). Dashed lines indicate weak non-covalent interactions, solid lines indicate strong non-covalent interactions. S-S, Disulphide bridges.

other anomalous but interesting property of these proteins is their unusual
behaviour at low pH values. The effect of low pH in the range of 5 to 1 on the
[o]meric structure, spectral properties and conformation of α-globulin (Prakash
[and] Nandi, 1977), arachin (Prakash and Narasinga Rao, 1986), brassin (M) (Kishore
[Kum]ar Murthy and Narasinga Rao, 1984), poppyverin (Srinivas, 1984) and
[gua]nthin (Sripad, 1985) has been reported. In the pH range 5 to 3 the proteins
[asso]ciate and below pH 3, they reaggregate. The proteins have also been shown to
[unfol]d between pH 5 and 3 and refold below pH 3. These proteins are made up of
[acidi]c and basic subunits and it is possible that below pH 3, the acidic and basic
[subu]nits reassociate (possibly into a different molecule) because of charge effects
[dicta]ted by pH. The reassociation may also be due to entropically driven hydro-
[phob]ic interaction, as has been shown for α-globulin (Prakash and Nandi, 1977). The
[disso]ciation and reassociation in acid pH can be represented schematically as

$$\downarrow \text{pH} < 3$$

$$\xrightarrow{\text{pH} < 3} \left\{ \begin{array}{l} \text{Aggregates to} \\ \text{high } M_r \text{ *protein.} \end{array} \right.$$
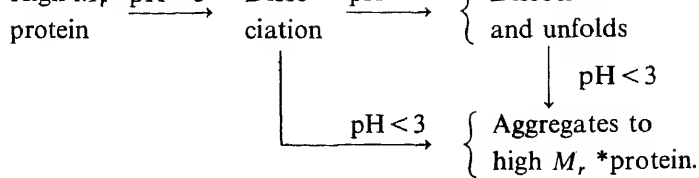
The reassociated molecule may be entirely different from the original molec protein even though it has been shown to have a sedimentation coefficient of (Prakash and Nandi, 1977). The results suggest that the forces which hold subu together in α-globulin, arachin, brassin (M), poppyverin and helianthin may be same. Also, the mechanism of reassociation must be very similar, since in all cases, reassociation results in a species of nearly the same sedimentation coeffic *i.e.*, the 11S component.

Dissociation-denaturation

The high M_r proteins behave very similarly when they are treated with denatura such as urea, guanidine hydrochloride (GuHCl) and SDS. Although vari intermediates are produced in the course of dissociation, the end product appear be a denatured 2S molecule (Prakash, 1985). The dissociation and denatura involve more than a single step as suggested by the intermediates produced different denaturant concentrations. All of them seem to have the same conforma namely, random coil, since intrinsic viscosity in 6 M GuHCl is close to tha random coil proteins and no ordered structures are present under these conditi However, α-globulin appears to be more asymmetric than other high M_r protein 6 M GuHCl (Prakash, 1985).

These results indicate a certain similarity in the oligomeric structure of the high proteins, and the following pattern:

$$\text{high } M_r \text{ proteins} \rightleftharpoons \text{Dissociation} \rightleftharpoons \text{Denaturation}$$
$$\downarrow \qquad\qquad\qquad |$$
$$\text{high } M_r\text{* protein} \leftarrow \text{Reassociation} \longleftarrow$$
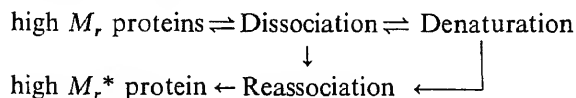
Quaternary structure

These data on association-dissociation, dissociation-reassociation and dissociat denaturation support the modified model of the hexamer sandwich, shown in figu The heterohexamer model for the high M_r oilseed proteins can explain the phys chemical properties of the proteins.

Based on this model, some features of association-dissociation and denatura can be explained. According to the model, the protein molecules are made up of hexamers. In this structure the stabilizing force is non-covalent interact especially entropically-driven hydrophobic interaction similar to that in nu acid stacking. Minor variations in pH, ionic strength, nature of ions etc.,

higher temperature ($<50°C$) favours association into the 11S form. Above 50°C dissociation may occur because of a totally different mechanism. The reaction may not proceed to unfolding of subunits because the basic and acidic subunits are held together by a much stronger covalent bond, *i.e.*, the S-S bond. The formation of the 7S trimer could also be facilitated by low concentration of urea, GuHCL or SDS, which destabilize the hydrophobic interactions between the monomers (acidic + basic subunit). This results in a situation where the non-covalent interactions between the trimers in the hexamer molecule are also destabilized. It could also mean that the nature of non-covalent interaction between the monomers in the trimer and the trimers in the hexamer could be different (figure 1, solid lines and dashed lines). At 8 M urea, 6 M GuHCL or 1×10^{-2} M SDS, the monomer or the 2S form is stabilized since all non-covalent interactions cease to exist at these concentrations of the denaturants. If a reducing agent is present, these monomers could further dissociate into acidic and basic subunits and would exist as completely unfolded polypeptide chains (figure 1). On the other hand if there is no reducing agent then the monomer would denature to a single molecule containing an S-S bond increasing the asymmetry of the uncoiled molecule (figure 1).

On the basis of the hexamer sandwich model, one can explain the unusual effect of acidic pH on the high M_r proteins. It is conceivable that as the pH is lowered from pH 7 to 4 hydrophobic interactions are weakened because of the increase in the concentration of H^+. Hence the molecule dissociates to a 2S component. From spectral data it is known that at this pH 4 aromatic groups, probably the contact groups between the subunits, are exposed. The increased energy of interaction may lead to the reassociation of these polypeptide chains rather uniquely since the hexamer is stabilised by noncovalent interactions.

Summarising, the proposed model has 4 main features. They are (i) area of association between acidic and basic subunits originates probably from the same precursor polypeptide chain (*i.e.*, the disulphide linkage area) (Pernollet and Mosse, 1983; Prakash and Narasinga Rao, 1986), (ii) the monomers form trimers by noncovalent association where each acidic (or basic) subunit associates with two basic (or acidic) subunits (Pernollet and Mosse, 1983), (iii) the trimer non-covalently associates with another trimer forming the hexamer and (iv) the non-covalent interactions within and between the trimers appear to be different from one another. This probably is the deciding factor in the stabilization of the native structure of the high M_r protein.

Biological significance

It is believed that the main function of the high M_r proteins in oilseeds is to serve as storage proteins. Recently, they have also been considered as secretory proteins (Pernollet and Mosse, 1983). These proteins are hydrolysed to amino acids during germination to create a large amino acid pool for synthesizing new proteins and to serve as a readily available nitrogenous source to facilitate growth of the plant. It is conceivable that the subunits are held together by weak forces ($\Delta F < 1$ Kcal/mol at

by successive planes of polypeptides joined by glutamyl residues (Pernollet and Mosse, 1983). This may be one reason why the high M_r proteins are rich in glutamic acid.

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analogues of leucine-methionine-enkephalin†

K. M. SIVANANDAIAH*, S. GURUSIDDAPPA and
D. CHANNE GOWDA

Department of Chemistry, Central College, Bangalore University, Bangalore 560 001, India

Abstract. Nine analogues of the opioid pentapeptides leucine-/methionine-enkephalinamide, involving replacement of amino acid at position 5 or amino acids at positions 2 and 5, have been synthesized by the solid phase method using mainly 9-fluorenylmethyloxycarbonyl amino acid trichlorophenyl esters in the presence of 1-hydroxybenzotriazole, the solid support being the Merrifield resin. All the analogues were effective in inhibiting the electrically stimulated contractions of the guinea pig ileum (*in vitro*) and one of them, tyrosyl-D-norvalyl-glycyl-phenylalanyl-methioninamide was found to be about 82 times more active than morphine. They also exhibited analgesic activity as well as antidiarrhoeal activity in mice (*in vivo*).

Keywords. Fmoc-amino acid active esters; Merrifield resin; enkephalin analogues; biological activity; structure-activity studies.

...luction

...art of our study of structure-activity relationships, a few analogues of ...halin, resulting from the single or multiple replacements of amino acids at ...ons 1, 2 and 5 of the natural sequence, Tyr-Gly-Gly-Phe-Leu/Met, were ...ted by us earlier (Sivanandaiah *et al.*, 1985). In continuation of these studies, ...nalogues were obtained by replacement of the amino acid at position 5 only ... more by replacement of amino acids at both positions 2 and 5. The biological ...ties of these analogues were studied.

...rials and methods

...he amino acids used except glycine are of L-configuration unless otherwise ...ied. Melting points were determined using Leitz-Wetzlar melting point ...ratus and are uncorrected. Thin-layer chromatography was carried out on silica ...ï (G. Merck, Darmstadt, West Germany) plates using the solvent system, ...oform:methanol:acetic acid (40:5:5), and the R_f value is designated as R_f ...). 9-Fluorenylmethyloxycarbonyl derivatives (Fmoc) (amino acids) were ...ned by the method of Chang *et al.* (1980) and their active esters according to ...andaiah and Gurusiddappa (1984). The completion of condensation and ...tection were monitored by Kaiser's test (Kaiser *et al.*, 1970).

... analogues (table 1) have been synthesized by .the solid-phase method ...rifield, 1963) employing the conventional Merrifield resin (G. Merck,

Table 1. List of peptides synthesized.

Peptide no.	Sequence
I	Tyr-Gly-Gly-Phe-D-Nva-NH$_2$
II	Tyr-D-Phe-Gly-Phe-D-Nva-NH$_2$
III	Tyr-D-Ala-Gly-Phe-D-Nva-NH$_2$
IV	Tyr-D-Val-Gly-Phe-D-Nva-NH$_2$
V	Tyr-Gly-Gly-Phe-Eth-NH$_2$
VI	Tyr-D-Ala-Gly-Phe-Eth-NH$_2$
VII	Tyr-D-Met-Gly-Phe-Eth-NH$_2$
VIII	Tyr-D-Ser-Gly-Phe-D-Met-NH$_2$
IX	Tyr-D-Nva-Gly-Phe-D-Met-NH$_2$

Darmstadt, West Germany). The peptide chain was built using Fmoc amino
active esters in the presence of 1-hydroxybenzotriazole (HOBt) following
protocol reported earlier (Sivanandaiah *et al.*, 1985). Fmoc group was remove
treatment with the less expensive diethylamine instead of the usually empl
piperidine. The introduction of Ser, however, was effected by dicyclohexylcarbodii
HOBt method. The N-terminal amino acid Tyr was introduced as butoxycarb
(Boc)-Tyr-trichlorophenyl ester (OTcp). The protected pentapeptides were
released from the resin by ammonolysis and the Boc group was cleaved fron
protected peptides by treatment with 98% formic acid in the presence of anisole
peptide salts were neutralised by treatment with IRA-400.

The synthetic peptides (I-IX) were assayed for their ability to inhibit the electr
induced contractions of the guinea pig ileum (GPI) (Kosterlitz and Watt, 1964)
analgesic and antidiarrhoeal activities of the analogues were studied in albino
using Eddy's hot plate test (Eddy, 1932) and charcoal meal test (Lenaerts, 1
respectively.

Results and discussion

The time required for the completion of coupling of each amino acid is given in
2 and the yields of protected and free pentapeptides are given in table 3. The ph
constants, analytical data and results of amino acid analyses are listed in tables
5. The relative potencies (GPI, analgesic and antidiarrhoeal) of these analogue
shown in tables 6 and 7.

Table 2. Reaction time for each amino acid residue.

Amino acid residue	Time (min)
Fmoc-Phe-OTcp	60
Fmoc-Gly-OTcp	60
Fmoc-D-Phe-OTcp	60
Fmoc-D-Ala-OTcp	50
Fmoc-D-Val-OTcp	90

Table 3. Yields of protected and free peptides.

Peptide	Yields (%)	
	X = Boc*	X = H
X-Tyr-Gly-Gly-Phe-D-Nva-NH$_2$	75	83
X-Tyr-D-Phe-Gly-Phe-D-Nva-NH$_2$	73	82
X-Tyr-D-Ala-Gly-Phe-D-Nva-NH$_2$	74	83
X-Tyr-D-Val-Gly-Phe-D-Nva-NH$_2$	75	81
X-Tyr-Gly-Gly-Phe-Eth-NH$_2$	68	78
X-Tyr-D-Ala-Gly-Phe-Eth-NH$_2$	71	80
X-Tyr-D-Met-Gly-Phe-Eth-NH$_2$	65	77
X-Tyr-D-Ser-Gly-Phe-D-Met-NH$_2$	74	78
X-Tyr-D-Nva-Gly-Phe-D-Met-NH$_2$	72	79

*Overall yield based on the amount of amino acid (D-Nva or Eth or D-Met) esterified to the resin.

ssay

iological activities of these analogues reveal (table 6) that substitution of the acid at position 5 of enkephalin-amides by ethionine (Eth) or D-norvaline (D-leads to loss of activity (I and V). In accordance with earlier observations ey, 1980), the introduction of a D-amino acid residue in place of Gly^2 causes a d increase in potency. Substitution by D-Phe, D-Ala and D-Val at position 2 Nva^5-enkephalinamide (I) increases the activity 10 to 20-fold, whereas tution by D-Ala and D-Met at position 2 of Eth^5-enkephalinamide (V) leads to d 93-fold increase in potency, respectively. Similarly, in the case of D-Met^5-halinamide which has a potency of only 0·105 relative to Met-enkephalin, tution by D-Ser/D-Nva at position 2 leads to 24/789-fold increase in potency. ver, it is apparent from the above results and other available data that the tude of increase in activity depends on the nature of the C-terminal part of the ule. Accordingly, [D-Ala^2, Eth^5]-enkephalinamide (VI) is more active than the ponding Pro^5-enkephalinamide but less active than [D-Ala^2, Nva^5]-halinamide; further, [D-Met^2, Eth^5]-enkephalinamide (VII) is more active than he corresponding Nva^5- and Pro^5-enkephalinamides. The incorporation of D-n place of Leu^5 in D-Phe^2-, D-Ala^2- and D-Val^2-leucine enkephalinamides decrease in their activity. In the case of D-Ser^2 analogue, substitution by D-t position 5 leads to a 10-fold increase in activity; this peptide, however, is y less active than the Nle^5 analogue. The spatial orientation of the side chain is rucial for activity as suggested by the low activity of [D-Ala^2, D-Nva^5]-halinamide compared to [D-Ala^2, Nva^5]-enkephalinamide. Among the 9 etic analogues now reported in this paper, [D-Nva^2, D-Met^5]-enkephalinamide as been found to be the most potent. It is 82·82 times more active than ine whereas D-Met^5-enkephalinamide has an activity of only 0·105 relative to nkephalin.

Table 4. Physical constants and analytical data of protected peptides.

Peptides	MP (°C)	R_f	$C[\alpha]_D^{25}$ (c, 1; DMF)	Molecular formula	Elemental analysis	%C	%H	%N
Boc-Tyr-Gly-Gly-Phe-D-Nva-NH$_2$	158–160	0·32	−13°	$C_{32}H_{44}N_6O_8$	Calcd.	60·0	6·88	13·13
					Found	59·75	6·68	13·29
Boc-Tyr-D-Phe-Gly-Phe-D-Nva-NH$_2$	140–142	0·51	−3°	$C_{39}H_{50}N_6O_8$	Calcd.	64·11	6·85	11·51
					Found	64·03	6·95	11·40
Boc-Tyr-D-Ala-Gly-Phe-D-Nva-NH$_2$	164–166	0·33	−17°	$C_{33}H_{46}N_6O_8$	Calcd.	60·55	7·03	12·84
					Found	60·36	7·23	12·62
Boc-Tyr-D-Val-Gly-Phe-D-Nva-NH$_2$	186–188	0·55	−20°	$C_{35}H_{50}N_6O_8$	Calcd.	61·59	7·33	12·32
					Found	61·39	7·22	12·46
Boc-Tyr-Gly-Gly-Phe-Eth-NH$_2$	166–168	0·41	−18°	$C_{33}H_{46}N_6O_8S$	Calcd.	57·73	6·71	12·25
					Found	57·43	6·70	12·19
Boc-Tyr-D-Ala-Gly-Phe-Eth-NH$_2$	172–174	0·47	−16°	$C_{34}H_{48}N_6O_8S$	Calcd.	58·29	6·86	12·00
					Found	58·42	6·64	12·23
Boc-Tyr-D-Met-Gly-Phe-Eth-NH$_2$	148–150	0·42	−6°	$C_{36}H_{52}N_6O_8S_2$	Calcd.	56·84	6·84	11·05
					Found	56·62	6·66	11·16
Boc-Tyr-D-Ser-Gly-Phe-D-Met-NH$_2$	154–155	0·67	+2°	$C_{33}H_{46}N_6O_9S$	Calcd.	56·41	6·55	11·97
					Found	56·43	6·75	11·79
Boc-Tyr-D-Nva-Gly-Phe-D-Met-NH$_2$	206–208	0·52	−8°	$C_{34}H_{48}N_6O_8S$	Calcd.	58·82	7·00	11·76
					Found	58·88	6·94	11·79

DMF, Dimethylformamide.

Amino acid composition of protected peptides.

	Tyr	Gly	Phe	Nva	Ala	Val	Eth	Met	Ser
Gly-Gly-Phe-D-Nva-NH₂	1·00	2·04	1·08	0·88	—	—	—	—	—
D-Phe-Gly-Phe-D-Nva-NH₂	1·02	1·15	1·93	0·90	—	—	—	—	—
D-Ala-Gly-Phe-D-Nva-NH₂	0·92	1·02	1·00	1·06	0·96	—	—	—	—
D-Val-Gly-Phe-D-Nva-NH₂	1·04	1·10	0·94	0·95	—	0·97	—	—	—
Gly-Gly-Phe-Eth-NH₂	0·91	2·12	0·96	—	—	—	0·95	—	—
D-Ala-Gly-Phe-Eth-NH₂	0·89	1·09	0·93	—	1·08	—	0·94	—	—
D-Met-Gly-Phe-Eth-NH₂	1·06	1·14	0·98	—	—	—	0·91	0·90	—
D-Nva-Gly-Phe-D-Met-NH₂	1·08	1·11	1·00	0·93	—	—	—	0·92	—
D-Ser-Gly-Phe-D-Met-NH₂	1·09	1·05	1·04	—	—	—	—	0·99	0·95

Name of the compound	Relative GPI potency
Morphine sulphate	1
Tyr-Gly-Gly-Phe-D-Nva-NH$_2$ (I)	0·0226
Tyr-D-Phe-Gly-Phe-D-Nva-NH$_2$ (II)	0·3816
Tyr-D-Ala-Gly-Phe-D-Nva-NH$_2$ (III)	0·2969
Tyr-D-Val-Gly-Phe-D-Nva-NH$_2$ (IV)	0·1955
Tyr-Gly-Gly-Phe-Leu-NH$_2$	0·21[a]
Tyr-D-Phe-Gly-Phe-Leu-NH$_2$	2·38[a]
Tyr-D-Ala-Gly-Phe-Leu-NH$_2$	2·10[a]
Tyr-D-Val-Gly-Phe-Leu-NH$_2$	0·28[a]
Tyr-Gly-Gly-Phe-Eth-NH$_2$ (V)	0·0917
Tyr-D-Ala-Gly-Phe-Eth-NH$_2$ (VI)	2·4908
Tyr-D-Met-Gly-Phe-Eth-NH$_2$ (VII)	8·5416
Tyr-D-Ala-Gly-Phe-Pro-NH$_2$	1·60[b]
Tyr-D-Ala-Gly-Phe-Nva-NH$_2$	3·20[b]
Tyr-D-Met-Gly-Phe-Pro-NH$_2$	5·71[b]
Tyr-D-Met-Gly-Phe-Nva-NH$_2$	7·27[b]
Tyr-Gly-Gly-Phe-D-Met-NH$_2$	0·105[c,d]
Tyr-D-Ser-Gly-Phe-D-Met-NH$_2$ (VIII)	2·4827
Tyr-D-Nva-Gly-Phe-D-Met-NH$_2$ (IX)	82·8192
Tyr-D-Ser-Gly-Phe-Leu-NH$_2$	0·26[a]
Tyr-D-Ser-Gly-Phe-Nle-NH$_2$	3·10[a]

[a] Sivanandaiah *et al.* (1985).   [b] Mathur (1981).   [c] Morley (1980).
[d] Potency relative to Met-enkephalin (= 1).

Table 7. Relative analgesic and antidiarrhoeal potencies of enkephalin analogues.

Name of the compound	Relative Potency	
	Analgesic activity	Antidiarrhoeal activity
Morphine sulphate	1	1
Tyr-Gly-Gly-Phe-Met	0·003[a]	
Tyr-Gly-Gly-Phe-D-Nva-NH$_2$ (I)	0·6500	1·2132
Tyr-D-Phe-Gly-Phe-D-Nva-NH$_2$ (II)	0·5833	0·8410
Tyr-D-Ala-Gly-Phe-D-Nva-NH$_2$ (III)	0·5610	0·9814
Tyr-D-Val-Gly-Phe-D-Nva-NH$_2$ (IV)	0·3433	1·0086
Tyr-Gly-Gly-Phe-Eth-NH$_2$ (V)	0·5626	0·6744
Tyr-D-Ala-Gly-Phe-Eth-NH$_2$ (VI)	0·5053	1·0963
Tyr-D-Met-Gly-Phe-Eth-NH$_2$ (VII)	0·6166	0·9631
Tyr-D-Ser-Gly-Phe-D-Met-NH$_2$ (VIII)	0·6610	0·7419
Tyr-D-Nva-Gly-Phe-D-Met-NH$_2$ (IX)	0·5553	0·8098
Tyr-D-Ala-Gly-Phe-D-Met-NH$_2$	16·6[a]	
Tyr-D-Ala-Gly-Phe-Met-NH$_2$	0·43[a]	

[a] Frederickson (1977).

Bioassays were performed at the Government College of Pharmacy,
Bangalore.

potencies were compared to that of morphine and all the synthetic analogues pos
50–66% of the potency of morphine. By this method Met-enkephalin has been fo
to have an activity of only 0·003 relative to morphine but its analogue, [D-Ala²

y decreased considerably. Further, we also observed that the introduction of place of Met in [D-Ala^2, Met^5]-enkephalinamide (reported to have an activity relative to morphine) increases the activity slightly. Among the 9 synthetic ues now reported, [D-Ser^2, D-Met^5]-enkephalinamide (VIII) has been found he most potent by this route of administration.

arrhoeal activity

eritoneal administration at a dose of 30 mg/kg showed good antidiarrhoeal . Among the 9 analogues tested, peptides I, IV and VI are more potent whereas es III and VII are almost equipotent compared to morphine. Analogue I, the otent in this assay, is the least potent in the GPI assay (table 7).

sion

the above results, it can be seen that a definite relationship seems to exist n the analgesic activity of the peptide and its potency in the GPI, and this has bserved by others also. Thus, the analogues (VI-IX) with high potency in the now good analgesic activity as well.

e enkephalin analogues, which are far less active compared to morphine in the ssay, show much higher potency as antidiarrhoeal agents, thus implying that eptors involved in the antidiarrhoeal activity of enkephalins may be different hose involved in the GPI assay. This has also been observed by others; the e, Tyr-Ile-Asn-Met-Leu, with a structure considerably different from that of nalin, has proved to be an effective antidiarrhoeal agent (Morley, 1980).

nces

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yridyl)methyl phosphoro-bis-triazolide as a new phosphorylating ent for internucleotide bond formation*

K. MISRA†, M. CHADDHA, A. DIKSHIT and R. K. SINGH

Nucleic Acids Research Laboratory, Department of Chemistry, University of Allahabad, Allahabad 211 002, India

Abstract. (α-Pyridyl)methyl phosphoro-bis-triazolide has been found to be a reagent of choice for phosphate protection in oligodeoxyribonucleotide synthesis. The reagent has been used successfully to phosphorylate all the four 5′-and N-protected deoxynucleosides. The resulting 3′-phosphorylated derivatives were found to be fairly stable as either triethyl ammonium salts or cyanoethyl derivatives. The phosphorylated derivatives were used in the preparation of the dimers T_pT and $d(A_pT)$ in solution phase and a tetramer, TTTT, and a hexamer, d(ATATAT), on solid phase using glass support. The method gave excellent yields. Considerably reduced condensation time (6 9 min) and practically no cleavage of the internucleotide bond during the removal of the group are the advantages.

Keywords. Phosphorylation; internucleotide-bond.

luction

ite tremendous progress in oligonucleotide synthesis, there still remain many ved problems. Improvements in oligonucleotide synthesis are being conti-sly introduced, including the use of better protecting groups at different nucleo- sites, mainly amino and phosphate groups. A number of groups have been in this laboratory for exocyclic amino protection (Mishra and Mishra, 1986; and Mishra, 1987; Mishra, K., Dikshit, A., Singh, R. K. and Chaddha, M., blished results). The phosphate protecting group must remain intact ghout the assembly of the oligonucleotide chain and therefore has to be selected great care. It should not be sensitive to acid or base catalyzed hydrolysis. The nt itself as well as the phosphorylated nucleoside should be stable, easily able, storable for prolonged periods and simultaneously reactive enough to with another monomer nucleoside without much activation. Thus, there are e problems of selectivity and compatibility in the use of these protecting groups. eral groups have been reported for the protection of the phosphate moiety, lly for use in the formation of internucleotide bond by the triester approach er et al., 1963; Reese and Saffhil, 1968; Letsinger and Mungall, 1970; van Boom 1976).

e phosphorodichloridates used earlier for phosphorylation led to the formation 3′ and 5′-5′ symmetrical dimers. In order to get rid of this problem, aryl

We have now phosphorylated all the 4 suitably protected deoxynucleosides, viz., 5'-O-dimethoxytrityl-thymidine (5'-O-DMTrT), 5'-O-dimethoxytrityl-N^6-benzoyl-2'-deoxyadenosine (5'-O-DMTr-N^6bz-2'-dA), 5'-O-dimethoxytrityl-N^4-benzoyl-2'-deoxycytidine (5'-O-DMTr-N^4bz-2'-dC), 5'-O-dimethoxytrityl-N^2-isobutyryl-2'-deoxyguanosine (5'-O-DMTr-N^2ibu-2'-dG) with the bistriazolide of 2-methylpyridyl phosphorodichloridate. These derivatives have been obtained in the form of their triethylammonium salts or cyanoethyl derivatives. The latter are sufficiently stable to be stored at 0°C for prolonged periods.

One of the major problems encountered during the removal of the phosphate protecting groups so far reported, has been the cleavage, to different degrees, of internucleotide bond (Cusack et al., 1973). During the removal of (α-pyridyl) methyl group by the attack of oximate anion, practically no cleavage has been observed.

Materials and methods

The deoxynucleosides were purchased from Yoshitomi Pharmaceutical Co., Japan. The base protection and tritylation was carried out as reported by Schaller et al. (1963). Dimethoxytrityl chloride, triazole, 1,1,3,3-tetramethyl guanidine, 4-nitrobenzaldoxime, 1-methylimidazole (MeIm), triisopropylbenzenesulphonyl chloride (TPSCl), mesitylenesulphonylnitrotriazole (MSNT) and long chain alkylamine controlled pore glass (LCAA-CPG) were purchased from Fluka, Buchs, Switzerland, Sigma Chemical Co., St. Louis, Missouri, USA, Biosearch, London, UK and Cruachem Chemical Co., Livingston, Scotland.

Solvents were duly purified prior to use. Thin-layer chrimatography (TLC) was carried out on silica gel G (E. Merck, Germany) plates and the plates sprayed with Ischerwood reagent, iodine and H_2SO_4 for location and differentiation of spots. Solid–phase synthesis was performed on a DNA double bench synthesiser (Omnifit Kit). Ultraviolet absorption was measured on a Hitachi 220 S spectrophotometer. High pressure liquid chromatography (HPLC) was carried out on LKB ultrapac (ODS Column, 9·4 × 250 mm). β-Cyanoethanol was prepared in the laboratory by reaction of 2-chlorethanol and KCN.

Preparation of 2-methylpyridine-N-oxide

The N-oxide of 2-methylpyridine was prepared by a method similar to that for pyridine-N-oxide (Vozza, 1962). The product, 2-methylpyridine-N-oxide was distilled at reduced pressure (138–140°C/15 mm Hg), yield 90 ml (91%).

Preparation of 2-acetoxymethylpyridine (Oae et al., 1962)

A mixture of acetic anhydride (100 ml) and 2-methyl pyridine-N-oxide (80 ml) was heated gently to 140°C and heating was continued for a further 5 min. After removing acetic acid and acetic anhydride from the reaction mixture, 2-acetoxymethyl pyridine was distilled at reduced pressure (90–92°C/5 mm Hg), yield 63·5 ml (79%).

Preparation of 2-hydroxymethyl pyridine

uced pressure (111–112°C/15 mm Hg). UV: λ_{max}(CH_2Cl_2) 285 nm; yield 57 ml

ration of (α-pyridyl)methyl phosphorodichloridate

lroxymethyl pyridine (93 ml; 1 mol) was treated with $POCl_3$ (360 ml; 4 mol)
ne reaction mixture was heated for 3 h in the presence of $AlCl_3$ (catalyst). The
ct was distilled at reduced pressure 120°C/12 mm Hg), yield 47 ml (50%).
product was confirmed by its alkaline hydrolysis. After hydrolysis, starting
ol, 2-hydroxymethylpyridine, was recovered, R_f 0·90 (C_6H_6/CH_3OH; 8·5:1·5,
dentical with the authentic sample.

ration of (α-pyridyl)methyl phosphoro-bis-triazolide

Triazole (13·35 g, 200 mmol) was dissolved in THF (500 ml). To this was
(α-pyridyl)methyl phosphorodichloridate (12 ml, 75 mmol) and triethyl-
(24·40 ml, 175 mmol). After shaking the reaction mixture for a few min,
ous white precipitate of triethylammonium chloride appeared. The precipitate
ltered off and the filtrate was directly used for the phosphorylation of 5′- and
tected deoxynucleosides.

ration of triethylammonium- 5′-O-DMTrT-3′-O-(α-pyridyl)methyl phosphate

DMTrT (545 mg, 1 mmol) was taken in dry THF (15 ml) and (α-pyridyl)methyl
horo-bis-triazolide (1·5 mmol) and 1-methylimidazole was added to the
on flask. Reaction was complete in 20 min as checked by TLC. The reaction
quenched by adding aqueous triethylammonium bicarbonate (200 ml, 1 M,
3) and then extracted with CH_2Cl_2 (2 × 15 ml). The organic layer was dried
odium sulphate and then evaporated *in vacuo* to a gum.
gum was subjected to silica gel column chromatography and elution was
d out with CH_2Cl_2/CH_3OH in increasing polarity with 1% $(C_2H_5)_3N$.
ions showing absorption at 270 nm were pooled and evaporated to dryness *in*
. The residue was dissolved in CH_2Cl_2 (2 ml) containing 1% $(C_2H_5)_3N$, and the
ct was precipitated by dropwise addition of this solution to vigorously stirred
ntane (50 ml). The white precipitate was collected by centrifugation at 4°C and
d with pentane. The pure product was dried over KOH and stored in a sealed
iner at low temperature, R_f 0·40 (CH_2Cl_2/CH_3OH; 9:1, v/v), UV: λ_{max}
Cl_2) 270 nm, yield 730 mg (90%).
nfirmation of the phosphorylated unit was carried out by total deprotection
T_p (nucleotide) was obtained in place of T (nucleoside) as confirmed by direct
arison with authentic samples chromatographically and spectroscopically.
ilarly, phosphorylated derivatives of 5′-O-DMTr-N⁶bz-2′-dA (R_f 0·58,
Cl_2/CH_3OH; 9:1, v/v, λ_{max}280 nm), 5′-O-DMTr-N⁴ bz-2′-dC (R_f 0·50, CH_2Cl_2/
OH; 9:1, v/v, λ_{max}305 and 261 nm), 5′-O-DMTr-N² ibu-2′dG (R_f 0·56, CH_2Cl_2/
OH; 9:1, v/v, λ_{max}278 and 262 nm) were also prepared.

by TLC (CH$_2$Cl$_2$/CH$_3$OH; 9·5:0·5, v/v). After completion of the reaction, β-cya
ethanol (20 mmol) was added to the flask and the mixture kept for 1 h ur
vigorous stirring. The reaction mixture was evaporated to a gum *in vacuo*. This
then dissolved in CH$_2$Cl$_2$, washed with 0·1 M NaH$_2$PO$_4$ (3 × 20 ml) and then v
water (2 × 20 ml). The organic part was dried over Na$_2$SO$_4$, evaporated to a g
and subjected to silica gel column chromatography eluting it with CH$_2$Cl$_2$/CH$_3$
in the presence of 1% (C$_2$H$_5$)$_3$N. Fractions were monitored at 270 nm and po
appropriately. The solution was evaporated to dryness *in vacuo* dissolved in CH$_2$
(5 ml). The product was precipitated with a mixture of dry ether/petroleum e
(50 ml, 2:3, v/v), R_f 0·60 (CH$_2$Cl$_2$/CH$_3$OH; 9·5:0·5, v/v), UV: λ_{max}(CH$_2$Cl$_2$) 270
yield 624 mg (84%).

Similarly, cyanoethylated derivatives of the other protected nucleosides were
prepared.

Removal of (α-pyridyl)methyl group

Triethylammonium salt of 5′-O-DMTrT-3′-O-(α-pyridyl)methyl phosphate
mmol) was treated with 1,1,3,3-tetramethylguanidinium salt of 4-nitrobenaldox
(0·3 mmol). Reaction was followed by TLC. Complete deprotection was achie
in 20 min.

Preparation of dimers, T_pT and d(A_pT)

The dinucleotides T_pT and d(A_pT) were prepared by condensing appropriate u
and co-evaporated to dryness *in vacuo* with the respective solvents, under va
reaction conditions (table 1). In condensation reactions, 1·5-fold molar excess c
component over 5′-OH component and 3-fold molar excess of TPSCl and M
each relative to P-component were used. After the appropriate time, water (5 ml)
added to the ice-cooled reaction mixture. The reaction mixture was extracted
CH$_2$Cl$_2$, washed with Na HCO$_3$ (0·1 M), dried over sodium sulphate and evapor
to a gum *in vacuo*. The dimers were purified by silica gel column (20 × 2

Table 1. Preparation and characterisation of dimers T_pT and d(A_pT).

3-Phosphate unit	Units 5′-OH unit	Solvent	Condensation Condensing reagent	Condensation time (min)	R_f^*	Yie (%
5′-O-DMTr-T-3′-O[(α-Pyridyl)-methyl]-phosphate	T-3′-OAc	Pyr	TPSCl	30	0·3	80
5′-O-DMTr-N-bz-dA-3′-O[(α-pyridyl)methyl]-phosphate	T-3′-O-Ac	Pyr	TPSCl+MeIm	9	0·33	84
		CH$_3$CN	TPSCl+MeIm	6	0·33	89

ence of 1% triethylamine. Fractions were monitored at 270 and 280 nm, respectively, and fractions containing the desired product were pooled and evaporated to dryness *in vacuo*.
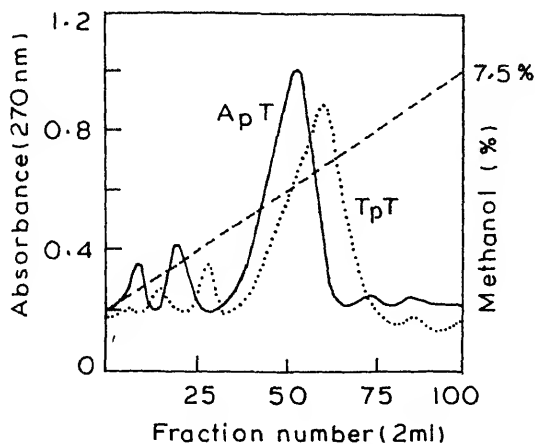
Figure 1. Column chromatography of fully protected dinucleotides.

The dry residue obtained above was treated with 1,1,3,3-tetramethylguanidinium of 4-nitrobenzaldoxime (0·23 mmol) in dioxane/water (1:1, v/v) for 20 min. The reaction mixture was evaporated to dryness and treated with ammonia (40%, 2 ml) 4 h to remove acetyl and benzoyl groups. The mixture was again evaporated to dryness *in vacuo* and treated with CH_3COOH (80%, 2 ml) at room temperature for min. The reaction mixture was evaporated to dryness and the residue was subjected to reversed phase silica gel column (10×2 cm) chromatography (figure 2) using with acetone/water in increasing polarity. The dimers were obtained in good yields as determined by trityl estimation (table 1).

In order to confirm the nature of the dimers, an aliquout from each was subjected hydrolysis with conc. NH_3 at 150°C for 24 h. The products, T_p and T, in the case T_pT and A_p and T in the case of $d(A_pT)$, were confirmed chromatographically and spectroscopically by direct comparison with authentic samples.
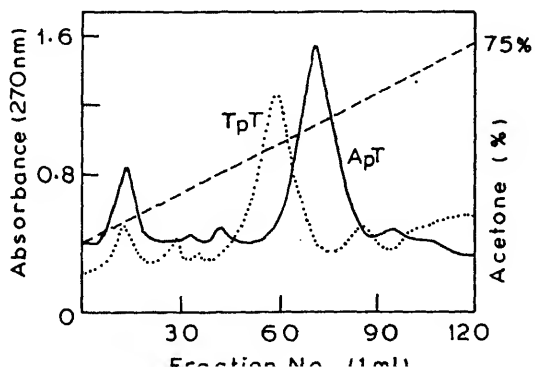
To 5'-O-DMTrT (543 mg, 1 mmol) dissolved in pyridine (5 ml), triethylamine (0·4 ml) and succinic anhydride (110 mg, 1·1 mmol) were added. The mixture was kept at room temperature for 12 h and then applied to a Dowex-50 (pyridinium form) column (10 × 2 cm) and eluted with pyridine/water (1:4, v/v). The eluate was evaporated to dryness *in vacuo*, dissolved in CH_2Cl_2 (0·5 ml) and purified on a silica gel column (6 × 2 cm). Elution was first carried out with CH_2Cl_2 followed by C_2H_5OH/CH_2Cl_2 (3:97, v/v). Trityl and sugar positive fractions of latter elution were pooled and the product precipitated with ether/pentane (3:2, v/v). R_f 0·26, (CH_2Cl_2/CH_3OH; 9:1, v/v), yield 476·6 mg (74%).

Preparation of tetramer TTTT and hexamer d(ATATAT) on solid support

The compound 5'-O-DMTrT-3'-O-succinate was linked to the solid support LCAA-CPG (Gough *et al.*, 1981). Loading was found to be 35 μmol g^{-1} as estimated by trityl analysis (Gait *et al.*, 1980). This derivatised support was used for the synthesis of tetramer and hexamer.

Functionalised support was taken in both columns (100 mg each) of the DNA bench synthesiser and the wash cycle run in the following order:

	Min
3% TCA in CH_3CN-CH_2Cl_2 (7:3, v/v)	3
CH_3CN-CH_2Cl_2 (7:3, v/v)	2
Coupling mix in CH_3CN-CH_2Cl_2 (7:3, v/v)	9
CH_3CN-CH_2Cl_2 (7:3, v/v)	2
CH_3CN-Ac_2O-MeIm (17:2:1)	3
CH_3CN-CH_2Cl_2 (7:3, v/v)	2

The cycles of wash and addition of incoming nucleotide unit (28 μmol) were carried out until the required chain length of oligomers was obtained. After the final coupling reaction, the support was washed with CH_2Cl_2, MeOH and ether. The support was taken out of the column and dried.

Deprotection and isolation of oligonucleotides

The tetramer and hexamer linked to the support were treated with 0·5 M solution of 1,1,3,3-tetramethylguanidinium-4-nitrobenzaldoxime in dioxane-water (1:1, v/v) for 16 h at room temperature. The support was filtered and the filtrates evaporated to dryness *in vacuo*. The residue was taken in 40% ammonia (5 ml). The flasks were sealed carefully and put in a thermostat bath at 60°C for 5 h. The reaction mixture was then evaporated to dryness *in vacuo*.

The above mass was taken in 0·1 M triethylammonium acetate and was analysed on reversed phase HPLC using C_{18} column. A gradient system of 20–30% acetonitrile in 0·1 M triethylammonium acetate over 15 min, was used for tritylated oligomers. The tritylated oligonucleotides were easily identified and isolated (figure 3). Fractions containing the desired sequences were concentrated and treated with 80% acetic acid (5 ml) to remove trityl group. After complete deprotection (30 min), the solution was concentrated and extracted with ether thoroughly. The fully
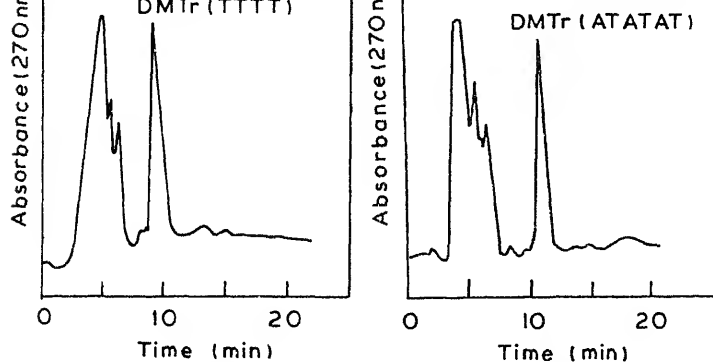
Figure 3. HPLC profiles of dimethoxytritylated tetramer (A) and hexamer (B).

...ugh C_{18} column using a gradient of 10–15% acetonitrile over 15 min at the rate ... $ml\ min^{-1}$ (figure 4).
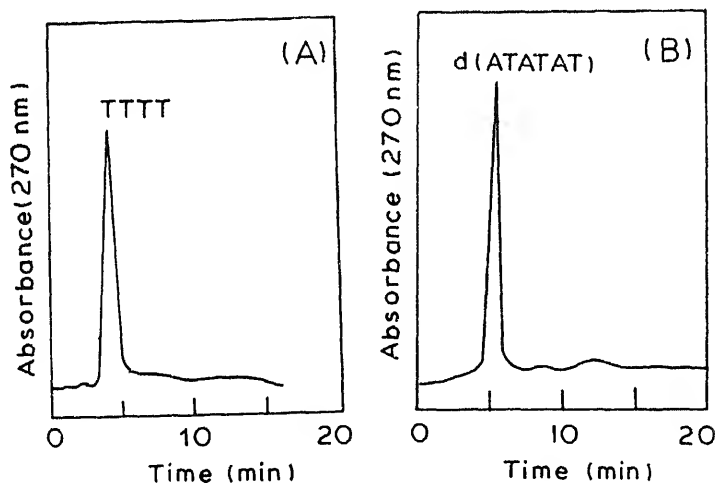
Figure 4. HPLC profiles of tetramer (A) and hexamer (B).

...lts and discussion

...reagent (α-pyridyl) methyl phosphoro-bis-triazolide (scheme 1) has been found ... a very promising phosphorylating agent, for all the 4 suitably protected ...ynucleosides. The phosphorylated derivatives (7, a–d) were obtained in approxi-...ly 90% yield and were isolated in the form of either triethylammonium salts or ...oethyl derivatives (scheme 2). The reactivity of the reagent was evident from the ... yields obtained which are comparable to those with o- and p-chlorophenyl ...phoro-bis-triazolides. The cyanoethyl derivatives were comparatively very ...e and could be stored at 0°C for prolonged periods without any decomposition

Scheme 1.

DMTr – Dimethoxytrityl

B – (a) N^6-benzoyl adenine-9-yl
(b) N^4-benzoyl cytosine-1-yl
(c) N^2-isobutyryl guanine-9 yl
(d) Thymine-1-yl
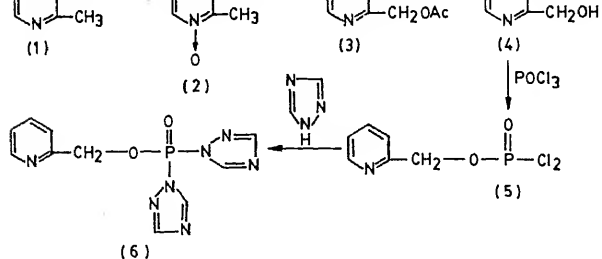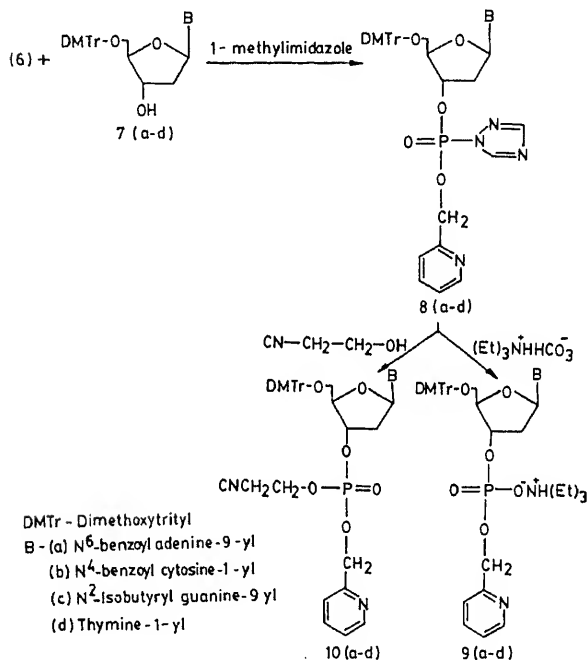
Scheme 2.

looked into and no detectable dimerisation was observed using 1·5-fold molar ex~~cess~~
of reagent and dry THF as solvent. This observation also supports the earlier rep~~ort~~
(Broka *et al.*, 1980).

In order to prove the efficiency of the phosphate protecting group, two di~~mers~~
T_pT and $d(A_pT)$ have been prepared in solution phase (scheme 3) using diff~~erent~~
conditions for the condensation reaction (table 1). In the preparation of ~~TpT~~
condensation time was reduced to 30 min using TPSCl as condensation reagen~~t (cf.~~
2–3 h in the case of *o*- and *p*-chlorophenyl derivatives; Broka *et al.*, 1980). During ~~the~~
preparation of the dimer, $d(A_pT)$, varied conditions for condensation were attem~~pted~~
and significant improvement was noticed. The condensation time was found t~~o be~~
~~6 min using acetonitrile as solvent and 9 min using pyridine; 1-methylimidazole~~

O NHEt₃ → $\overset{|}{O}\ NHEt_3$

(9)
+

TPSCl
MeIm

$O=\overset{|}{\underset{|}{P}}-O-CH_2-$ (pyridine)

B₂

O=C—CH₃
(12)

(i) oximate
(ii) NH₃ (40 %)
(iii) H⁺

B₂

HO

O

O=C—CH₃

(11)

B₁ = (a) thymine-1-yl
    (b) N⁶-benzoyl adenine-9-yl

B₂ = Thymine-1-yl

B₁

HO

O

O=P—O⁻

B₂

O

ÓH

(13)

Scheme 3.

The reagent was further checked for versatility of use in oligonucleotide synthesis using solid phase methodology. Two oligomers, TTTT and d(ATATAT) were prepared (scheme 4) in good yields (79 and 68%, respectively) using LCAA-CPG as the solid support. Synthesis was achieved by successive addition of fully protected nucleotide derivatives. At each step, 8-fold excess of P-component over the nucleoside capacity on the support was used. The coupling reagent MSNT and the catalyst MeIm were used in 3-fold and 6-fold molar excess respectively to P-component. In both cases, one-solvent procedure for rapid solid phase synthesis was used (Efimov et al., 1982).

The dimethoxytrityl group was removed after each coupling step using 3% trichloroacetic acid in a mixture of acetonitrile and dichloromethane. Trityl analysis (Gait et al., 1980) at each step suggested nearly a constant yield of approximately 94% (as in the case of shorter sequences). The repeated acid treatment did not cause any detectable depurination in the case of the hexamer as no peak corresponding to the base adenine was observed during HPLC purification.

The constant coupling yield suggested minimum of failure sequences or internucleotide chain scission. The percentage yield of di- and trimer in the case of tetramer and di-, tri-, tetra- and pentamer in the case of hexamer as suggested by HPLC analysis of the mixture after the removal of the group were found to be

Scheme 4.

congruous with the loss at each coupling step in their preparation (as proved b
trityl estimation). These results suggest the complete absence of internucleotide bor
cleavage or very insignificant cleavage, if any, during the removal of the group.

Thus the remarkable advantages of the reagent are its easy introduction with goo
yields, better stability of the derivatives, practically no cleavage of the internucleoti
bond during its removal and reduced coupling time with higher yields.

Acknowledgement

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Purification and characterisation of prolactin from sheep and buffalo pituitaries†

K. MURALIDHAR*, NEERAJA CHADHA and RITA KOHLI

Laboratory for Bio-chemistry, Department of Zoology, University of Delhi, Delhi 110 007, India

Abstract. A study of the problem of structural variants of proteins and their relative contribution to the expressed immunological and biological activity has been initiated using sheep and buffalo prolactins as models. The feasibility of obtaining immunologically and biologically active prolactin in high yields from the discarded 'acid pellet' of sheep and buffalo pituitaries has been demonstrated. This permits use of the same batch of glands for purifying lutropin, follitropin and prolactin as side fractions. The major component in preparations of buffalo prolactin has a molecular size of 24 kDa. The preparations were active in a radioligand binding inhibition assay and in a rat liver based radioreceptor assay. Charge and size isomers of sheep prolactin and buffalo prolactin have been observed. The reference sheep prolactin did not, in preliminary work, give any indication of being glycosylated. However radioactive sulphate was found to be incorporated into prolactin-rich fractions of sheep and buffalo pituitaries *in vitro*. By physico-chemical and immunochemical criteria the [^{35}S]-labelled material was similar to standard reference prolactin. The structural implications of sulphation have been probed.

Introduction

The pituitary polypeptide hormone prolactin (PRL) plays an important but enigmatic physiological role in all vertebrates from fish to mammals (Ensor, 1978). Over 80 biological effects have been ascribed to PRL (Nicoll *et al.*, 1986). Inspite of an astonishingly large body of information on its structure, biosynthesis, secretion and physiological action, nothing is understood regarding either the mechanism of its action or the relation of its structural domains to biological effects (Nicoll *et al.*, 1986).

A number of reports have claimed detection of multiple forms of PRL. These reports have identified size differences among these structural variants. Recently, Lewis *et al.* (1984) reported the detection and purification of a glycosylated form of ovine PRL. However, a detailed study of the origin and relative contribution of the different polymorphic forms of PRL to its expressed biological and immunological activity has not been made till now.

We have recently initiated a programme of study of PRL from buffalo. Our aim in this study was to relate the structural domains of buffalo PRL to physiologically and immunologically important epitopes especially from an evolutionary perspective. The bewildering variety of its actions—osmoregulation in fish, somatotropic action in amphibians, parental and migratory behaviour in birds, growth and tissue regeneration promotion in reptiles and luteotropic and luteolytic effects in mammals—

Materials and methods

Hormones, chemicals, antisera and animals

Sheep PRL for radioiodination and rabbit anti-ovine PRL serum for radioimm
assay (RIA) were obtained through the courtesy of Dr. S. Raiti, National Institu
Diabetes and Digestive and Kidney Diseases (NIDDK, NIH) Bethesda, Maryl
USA. Enzyme (penicillinase)—human PRL conjugate, rabbit anti-human PRL
goat anti rabbit γ-globulin were provided by Dr. G. L. Kumari of the Nati
Institute of Health and Family Welfare, New Delhi. Ovine PRL (averag
35 IU/mg), reference marker proteins for sodium dodecyl sulphate (SDS)-polya
amide gel electrophoresis (PAGE), bovine serum albumin (BSA), Sephadex-
DEAE Sephadex-A50, acrylamide, bis acrylamide, TEMED, Freund's com
adjuvant, ammonium persulphate, Coomassie blue R-250, concanavalin A (Co
Sepharose and α-methyl-D-mannoside were purchased from Sigma Cher
Company, St. Louis, Missouri, USA. Carrier-free Na ^{125}I and carrier-free ^{35}S
were obtained from Bhabha Atomic Research Centre, Bombay. PPO and dim
POPOP were purchased from SISCO research laboratories, Bombay. The W
reference standard ovine PRL (22 IU/mg) was obtained through the Nati
Institute of Biological Standards, England. Adult rats employed in this study we
Holtzman strain, and were maintained in our colony under standard 14 h L : 10
schedule. They were given food (Hindustan Lever Ltd., Bombay) and wate
libitum. Male albino rabbits weighing 2·5 kg were purchased from Maulana ⁄
Medical College, New Delhi. All chemicals used in this study, unless other
mentioned were of Excelar/GR grade.

Pituitary incubations in vitro

Incubations for examining $^{35}SO_4^{2-}$ incorporation were carried out using minc
sheep and buffalo pituitaries collected fresh from a local abattoir. All incuba
were done at 37°C in a metabolic shaker and in modified KRBG b
(Arunasmithasri et al., 1983). In sulphate incorporation studies $MgSO_4$ was repl
by $MgCl_2$. All media included amino acid and vitamin mixture as in Medium
(Morgan et al., 1950).

Purification of PRL

Glands were collected from sheep and buffaloes which were being slaughtere
a local abattoir. They were frozen in liquid N_2 within 1 h of slaughter and t
ported to the laboratory in liquid N_2 for processing. The procedures
adaptations of those of Papkoff et al. (1965), Li (1974), NaiSiang-Jiang and Will
(1965) and Bell et al. (1985) with a few modifications. Briefly, the glands were mi
and homogenised in 0·15 M (NH₄) SO in a Waring blender and the homog

...matants were used for the purification of lutropin (LH) and follitropin (FSH). In
...adaptation of the NaiSiang-Jiang and Wilhelmi (1965) procedure, chilled
...°C) alkaline ethanol (pH 9·5) was added to the pH 4 acid pellet and the two
...mixed well. The suspension was centrifuged. To the supernatant at pH 5·5 were
...d 2 volumes of chilled ethanol to precipitate enriched PRL (P-1 fraction). This
...pitate, collected by centrifugation, was dissolved and dialysed against weak
...onium bicarbonate and lyophilised. The material was then subjected to
...matography on DEAE Sephadex equilibrated with 0·01 M phosphate-0·0065 M
...e buffer, pH 7. The bound proteins were eluted with *a* step wise gradient of
... In the adaptation of the procedure of Li (1974), the pH 4 acid pellet was
...cted with acidic acetone followed by the precipitation of PRL with a further 5
...mes of chilled acetone. The precipitate was dissolved and subjected to
...ential isoelectric precipitation by gradual adjustment of pH (figure 1). The

DEAE Sephadex.
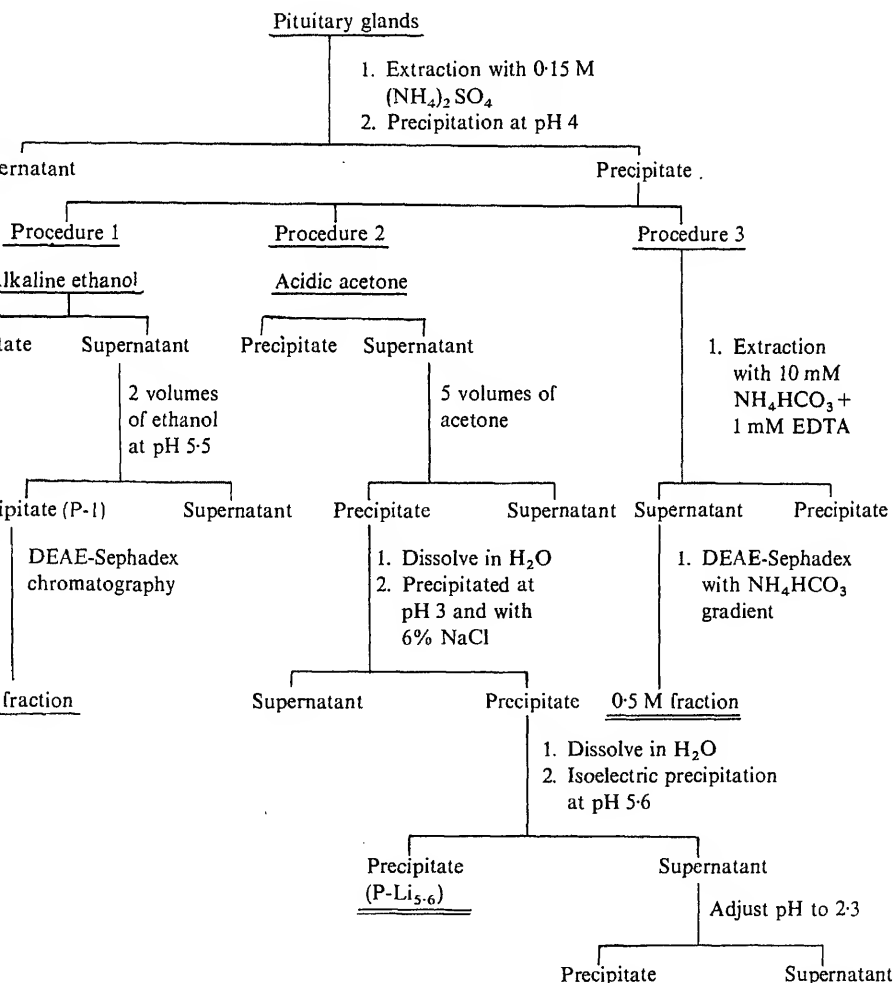
Disc electrophoresis

Electrophoresis under denaturing conditions was carried out essentially according
the procedure of Laemmli (1970) using 11% resolving gels. Disc electrophoresis
native gels was carried out according to the method of Davis (1964). Electrophore
transfer of proteins from gels onto nitrocellulose paper was carried out according
the procedure of Towbin *et al.* (1979) except that 80 mA current was used for 12 h a
Whatman No. 1 filter sheets were employed for padding. Immuno-staining v
carried out by the use of biotinylated second antibody and biotinylated peroxida
avidin system as described by Vector Laboratories, USA (Hsu *et al.*, 1981). A 1 : 1
dilution of the rabbit antibody (local) was used.

Con A-Sepharose chromatography

Affinity chromatography on Con A-Sepharose was performed according to
standard procedure of Bloomfield *et al.* (1978) at pH 6.

Antiserum

Antiserum to commercial ovine PRL (Sigma Chemicals, USA) was raised in rabl
essentially according to the procedure of Vaitukaitis *et al.* (1971).

Immunoprecipitation

Incubation of minces from sheep and buffalo pituitary glands (4–5/flask) was carr
out in 10–20 ml of modified KRBG in the presence of radioactive sulph
(200 μCi/ml). At the end of the incubation the tissue was homogenised and proces
as in the case of bulk preparation of PRL. The dialysed P-1 fraction (figure 1) v
taken for immunoprecipitations. ^{35}S-Labelled proteins from this fraction (1(
200 μg protein containing 2000–2500 cpm) were incubated with excess of rab
antiserum to PRL or normal rabbit serum for 1 h at 37°C and 3 days at 4°C. ⊺
buffer used was 0·01 M phosphate buffered saline, pH 7·5. The immunoprecipit
was collected by centrifugation at 3000 g for 15 min at 4°C, washed with chil
physiological saline by centrifugation, and dissolved in a small volume of 0·0;
NaOH. Aliquots were then taken for radioactivity measurement. In the case of
incubation medium, it was dialysed extensively and then lyophilised. The lyophili
powder was then subjected to immunoprecipitation as above.

Radioiodination and radioligand binding inhibition test

Radioiodination of ovine PRL (NIH standard) was performed essentially accordin

bit antiserum to ovine PRL (NIH product) at 1:25,000 initial dilution. Reference
ne) and buffalo PRL were used for competitive binding inhibition.

was carried out using rat liver homogenate as a source of PRL receptors
ntially according to the method of Taga (1982) with slight modifications. Briefly,
μl of rat liver homogenate, 100 μl of ^{125}I-labelled ovine PRL (80,000 cpm) and
μl of a serially diluted ovine PRL standard or the unknown sample were
bated in 0·025 M buffer, pH 7·6/10 mM CaCl$_2$/0·1% BSA at 37°C for 2 h
wed by the addition of 2 ml of the RRA buffer. The tubes (10 × 75 mm) were
rifuged in a Sorvall RC-2B centrifuge at 3000 g for 15 min at 4°C. The super-
nts were discarded and after wiping the sides free of adhering liquid, the
ts were counted for radioactivity in an ECIL type manual counter at 70%
iency. Suitable controls were included.

yme immunoassay

enzyme immunoassay (EIA) was performed according to a method standardised
e laboratory. Briefly 100 μl of anti human PRL serum (1:50,000 dilution) and
μl of reference human PRL (serially diluted, 100–4000 pg/tube) or the unknown
incubated for 16 h in 0·01 M phosphate-buffered saline, pH 7 containing 0·05%
, 0·1% sodium azide and 0·05% (v/v) Tween 20. At the end of the incubation, 100 μl
2000 diluted enzyme (penicillinase)—PRL conjugate (1 mg/ml stock) was added,
incubation continued for 6 h at 37°C. Then goat anti-rabbit γ-globulin serum
0 dilution) was added to all tubes followed by incubation for 36 h. The tubes
centrifuged and supernatants discarded. The substrate solution (1 mM)
wed 1 h later by the starch-I$_2$ reagent were added. After 10 min, the reaction was
inated by the addition of 5 N HCl (1 ml/tube) and the colour developed was
at 620 nm in a spectrophotometer.

ults and discussion

had initially examined the feasibility of purifying PRL from freshly frozen buffalo
itary glands. As there was no previous work on PRL from this animal species, we
ed procedures published for sheep, cattle and pigs (NaiSiang Jiang and Wilhelmi,
5; Li, 1974; Bell et al., 1985). We had earlier shown that the procedure of Papkoff
l. (1965) for the purification of ovine LH was applicable with a few modifications
buffalo pituitary glands (Arunasmithasri et al., 1983; Muralidhar and
endrakumar, 1986). In the course of the work on LH, the chance observation was
le that the acid pellet—the fraction usually discarded in the procedure employed
LH purification—was reacting positively with a specific rabbit antiserum to
e PRL (Neeraja Chadha et al., 1987). Surprisingly, the pH 4 acid pellet gave a

PRL from this fraction by simply applying the published procedures for pituitaries [of] sheep, cattle and pigs with a few modifications to the pH 4 acid pellet (figure [1]). Procedures 1, 2, 3 in the figure 1 refer to combination of the procedure of Papkoff [et] al. (1965) with those of NaiSiang Jaing and Wilhelmi (1965), Li (1974) and Bell et [al.] (1985), respectively. These preparations are referred to respectively as bu-P-W-P[-1], bu-P-Li and bu-P-B in table 1. The procedure of Bell et al. (1985) did not yield a[ny] immunoreactive material from the acid pellet of sheep pituitary glands but was use[ful] for the buffalo acid pellet. The procedures of NaiSiang Jiang and Wilhelmi (196[5]) and of Li (1974) were equally applicable to the acid pellets of sheep and buff[alo] glands. The material designated as P-1 was highly enriched in PRL as indicated [by] RIA estimatable PRL which was obtained in high yields (table 1). The combinati[on] of the procedures of Papkoff et al. (1965) and NaiSiang Jiang and Wilhelmi (196[5]) worked well for obtaining PRL from both sheep and buffalo acid pellet. It was, [for] buffalo and sheep pituitary glands, a single fractionation scheme which yielded L[H,] FSH and PRL as different fractions. NaiSiang Jiang and Wilhelmi (1965) w[ho] combined the classical scheme of Ellis (1961) with a few additional steps report[ed] good yields of PRL from ovine, bovine and porcine pituitary glands. However th[ey] did not report purification of LH and FSH also from the same batch of glands. [We] have established recently that their scheme of purification starting from Ellis (19[65]) fractionation procedure also yields highly enriched PRL from buffalo pituita[ry] glands (table 1, bu-E-W-P-1).

Table 1. Yield of PRL (immunoreactive) in the procedures using acid pellet as starting material.

Fraction	Oüchterlony test against rabbit a/s to ovine PRL	Yield (g/kg glands)
o-P-W-P-1	+	1·0–1·5
o-P-Li$_{5.6}$	+	0·4–0·5
bu-P-W-P-1	+	2·0–2·5
bu-P-Li$_{5.6}$	+	0·04–0·05
bu-P-Li$_{2.3}$	+	0·06–0·07
bu-P-B	+	0·16–0·18
E-W-bu-P-1	+	0·5–0·6

o, ovine; bu, buffalo; P, Papkoff et al. (1965) procedure; Li, procedure of Li (1974); B, procedure of Bell et al. (1985); W, Wilhelmi; E, Ellis (1965) procedure.

All the preparations were active in a radioligand binding inhibition test using [125I] labelled ovine PRL, rabbit anti-ovine-PRL serum and ovine-PRL as refere[nce] standard (table 2). Though they were tested at the same dose, the degrees [of] inhibition were different. As there was obviously no relation to purity (for exam[ple] the NIDDK sample is iodination grade but has only 66% activity), the difference[s]

[125]I-ovine PRL (NIDDK sample) and different PRLs (5 ng/tube level) were incubated with anti-ovine PRL serum and bound radioactivity measured.

Sample	$B/B_0 \times 100$
—	100
Sigma PRL	0
bu-E-W-P-1*	27·7
bu-P-Li$_{5\cdot6}$	33·4
NIDDK ovine PRL	33·7
bu-P-W-P$_1$-0·2 M fraction	63·0

*E, Ellis procedure. For other abbreviations see table 1.

degree of inhibition reflects the different avidities of the antiserum for these preparations. This in turn reflects the differences in antigenic epitope. If they indeed represent the *in vivo* situation, it would be extremely interesting. We are presently trying to develop a homologous RIA system for buffalo PRL. The buffalo pituitary PRL's (all the preparations) did not cross react with human PRL when tested in an EIA. The results are given in table 3. The absorbance values obtained for the

Table 3. Absence of cross reaction between buffalo and human PRL in an EIA.

Sample	Absorbance (620 nm)
Control (NRS)	2·016
Antiserum	0·285
Antiserum + 4 ng human PRL.	1·715
Antiserum + 100 ng buffalo PRL (bu-P-W-P-1 0·2 M)	0·229

different preparations of PRLs were around the same as that indicated in table 3 for bu-P-W-P-1$_{0\cdot2 M}$. Aston and Ivanyi (1985) in a study of human PRL using monoclonal antibodies have shown that two of them could bind both human and bovine/ovine PRL in a labelled antibody competition test. This would mean that even without selection by using ^{125}I-labelled ovine PRL for screening hybridoma supernatants containing secreted antibodies against human PRL, one can obtain cross-reaction between human and non-primate PRL. In our test we used a polyclonal antiserum, and it is possible that the cross-reacting antibodies were in too low a concentration to be detected in a competitive test.

The buffalo PRL preparations were also active in a rat liver based RRA (Taga, 1982). Figure 2 illustrates the results with reference PRL and with buffalo PRL prepared by the combination of the procedures of Papkoff et al. (1965) and Li (1974). The results also indicate that bovine growth hormone (GH) not exhibit significant activity in the range tested. Approximately 5000 ng of GH were needed to cause 50%
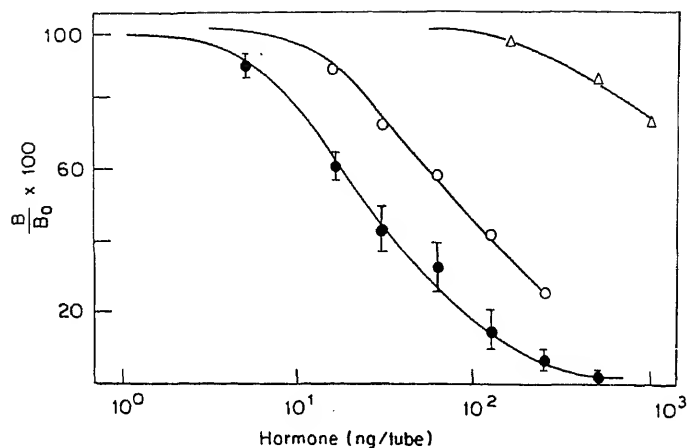
Figure 2. Radioreceptor assay for PRL using rat liver homogenate as a source of receptors. ($\bullet$), ovine PRL (Sigma); ($\bigcirc$), buffalo PRL (bu-P-Li$_{5.6}$); ($\triangle$), bovine GH.

less than 0·5% activity in this assay system. It also proves that although the purification of buffalo PRL was monitored by immunological activity, the preparation was biologically active in a receptor system specific to PRL and hence is not a cross-reacting substance.

It was possible to further purify the P-1 preparation by subjecting it to DEAE-Sephadex chromatography. A representative chromatographic profile for the bu-P-W-P-1 preparation is given in figure 3. It can be seen that PRL gets bound to the column and is eluted with 0·2 M NaCl.

Although we do not have evidence it is possible that there was a biologically active but immunologically inactive PRL in other fractions. Immunologically poor
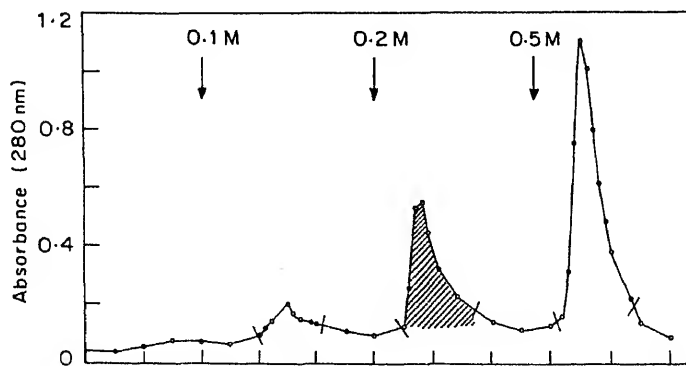
biologically very active forms of mouse PRL, for example, have been reported
ia and Baxter, 1979).

must also be mentioned that only the Papkoff-Wilhelmi procedure gave a PRL
h was eluted from DEAE Sephadex at 0·2 M NaCl. The procedure of Bell *et al.*
5) gave a PRL which was eluted from DEAE Sephadex only with
M NH_4HCO_3. It is possible that prolonged exposure to NH_4HCO_3 in this
edure caused some deamidation giving rise to more acidic forms of PRL.
vever, isoelectric focusing has not been done yet to substantiate this idea. In the
edure of Li (1974) also, while the sheep acid pellet gave us a PRL which
ipitated at pH 5·6, the buffalo acid pellet gave one at 5·6 (bu-P-Li$_{5.6}$) and
her immunoreactive PRL at pH 2·3 (bu-P-Li$_{2.3}$). These results indicate the
ible presence of charged isomers of PRL. Many reports in the literature do point
ich a situation based on electrophoretic analysis of PRL in native gels. We have
found that the reference standard PRL (Sigma) and our 0·2 M fraction of bu-P-
-l show multiple bands on electrophoresis in native gels (figure 4). However, by
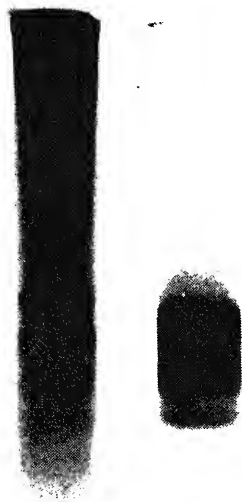
study of Graf et al. (1970) who isolated two forms of PRL, a normal and a faster mo[v]
ing band. Peptide maps showed that the two forms differed only in one peptide sp[ot.]
The difference was found to be attributable to Asn/Asp content. Whether deamid[a-]
tion occurs within cells or in the blood (enzymatic or otherwise) and if so, its physi[o-]
logical significance, are not known. Reports of altered immunological and recept[or]
binding properties of the deamidated form of mouse PRL, however, do exist in t[he]
literature (Haro and Talamantes, 1985).

In view of the report of Lewis et al. (1984) of the existence of glycosylated forms [of]
ovine PRL, we examined the commercial preparation of ovine PRL. We found [no]
evidence for the presence of glycosylated forms. Neither the hormone nor even t[he]
^{125}I-labelled ovine PRL was found to be retained on a Con A-Sepharose affin[ity]
column. Less than 1% of the loaded material was found retained. There was [no]
directly estimatable sugar in the dialysed preparation (Neeraja Chadha a[nd]
K. Muralidhar, unpublished results). It gave two closely moving Coomassie bl[ue]
stainable bands in SDS-PAGE indicating a difference in molecular size of ab[out]
1500–2000. The 0·2 M fraction from DEAE-Sephadex chromatography also gav[e a]
similar pattern (data not shown). However on immunoblotting after electrophor[etic]
transfer, both the bands reacted with the antibody (figure 5, left and right lanes).

Based on our previous experience with the glycoprotein hormone LH, metabo[lic]
studies were conducted to throw additional light on the problem of size variati[on]
due to glycosylation. We had earlier demonstrated by physico-chemical, immun[o-]
logical and biological criteria that pituitary LH has sugar-bound sulph[ate]
(Arunasmithasri et al., 1983; Rajyalakshmi et al., 1983; Muralidhar a[nd]
Rajendrakumar, 1986). A chance observation that significant radioactivity w[as]
present in the PRL rich fraction made us investigate this further. To our surprise [we]
found that pituitary minces from sheep and buffaloes incorporate $^{35}SO_4^{2-}$ from [the]
incubation medium into immunoprecipitable PRL-like material. Both tissue a[nd]
incubation medium yielded labelled immuno-precipitate (Rita Kohli et al., 1987). T[he]
material could be subjected to a fractionation scheme like that for purification [of]
PRL and radioactivity was still associated with purified PRL (Rita Kohli et al., 19[87]).

The ^{35}S-labelled proteins from the sheep pituitary tissue were extracted, purified [to]
the P-1 stage and then subjected to SDS-PAGE analysis. Parallel strips were ta[ken]
for staining, slicing and counting for radioactivity and also for slicing and extract[ion]
for immunodiffusion tests. From table 4 it is obvious that there are a number of $^{3[5}$S]
labelled proteins in the P-1 fraction. Two of these are of interest. They had molecu[lar]
size in the region of 25 kDa but differing by 1500–2000 (figure 5). Interestingly b[oth]
the proteins gave precipitin lines in the Ouchterlony test (figure 6). Protein from s[lice]
two (table 4) which had significant radioactivity did not react with anti-PRL ser[um.]
The Ouchterlony data was confirmed by Western blot analysis (figure 5, mid[dle]
lane). These observations raise more questions rather than provide answers.

The nature of the linkage between sulphur and the PRL peptide backbone is [not]
clear. A number of possibilities exist: (i) both these bands (figure 5, middle la[ne]
contain PRL with sugar-SO_4, (ii) both the forms are tyrosine-SO_4-containing P[RL]
with the larger protein having a chemical modification leading to the size incre[ase]
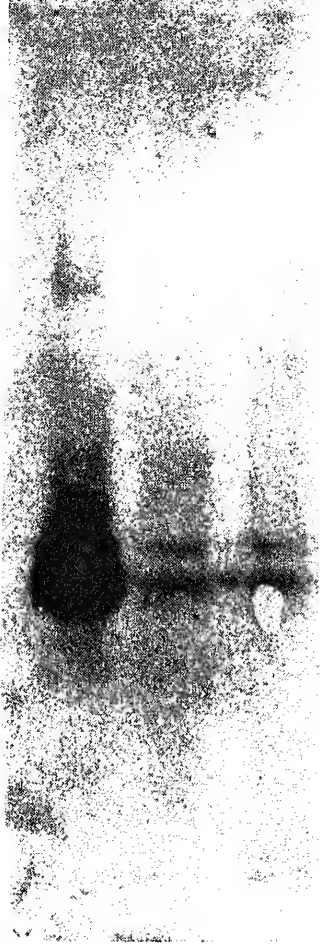
Figure 5. Western blot picture of standard PRL (left lane), ^{35}S-labelled P-1 (middle lane) and 0·2 M fraction from DEAE-Sephadex chromatography (right lane). These were subjected to SDS-PAGE and then transferred to nitrocellulose sheet.

adsorption on Con A Sepharose, a commercial preparation of sheep PRL was used. The reason for invoking the presence of sugar-SO$_4$ is that the ^{35}S-labelled immuno reactive proteins may differ from the commercial preparation. However, none of these alternatives point to the existence of the non-glycosylated and non-sulphated PRL and 24 kDa known for over 40 years (Li, 1980). A 20 kDa PRL has been reported to be present in human pituitary extracts (Aston *et al.*, 1984). Aston and Ivanyi (1985) have shown that even affinity purified human PRL, *i.e.*, PRL purified on monoclonal antibody affinity columns, gives multiple bands of Western blots. Two bands, at 26 and 24 kDa positions, were predominant. Minor bands at 18,

fraction).

Sheep pituitary minces were incubated *in vitro* with $^{35}SO_4^{2-}$ and at the end of the incubation the tissue was fractionated to yield P-1 fraction. This was subjected to SDS-PAGE as per the procedure of Laemmli (1970) using 11% preparative gels.

Slice No.	Radioactivity (cpm)*
1	512
2	1148
3	518
4	394
5	258
11	80
13	5584
14	2626
15	118

*After subtracting background radioactivity.
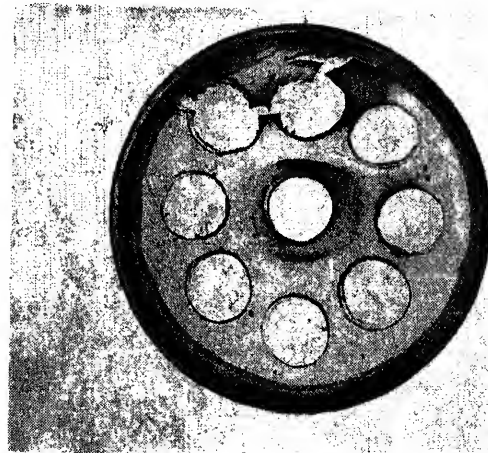
Figure 6. Oüchterlony immunodiffusion test results. The central well had rabbit anti PRL serum and the surrounding wells had materials as follows: 12-O' clock well, standard ovine PRL; 3-O' clock well, slice no. 13 extract; 6-O' clock well, slice no. 14 extract. All other wells had extracts from control slices taken arbitrarily from other portions of SDS-PAGE slab gel.

analysis. Bovine PRL gave a less intense 26 kDa band. There have been other reports of the existence of size variants of PRL. These include aggregates (Squire *et al.*, Nyberg *et al.*, 1980; Niall, 1981), monomers with chemical modifications

contributions of these forms to the biological activity of the hormone have not been
fully investigated. On the other hand, these forms may be experimental artifacts (*e.g.*
due to lyophilisation). However, size variants of proteins are known to arise as a
result of alternative processing of mRNA, post-translational modifications, or
proteolytic in circulation.

In conclusion, it has been possible to obtain highly enriched preparations of PRL
from the discarded acid pellets of sheep and buffalo pituitary glands. It was possible
to obtain the PRL in a homogeneous form. Further purification of the PRL prepa-
rations from sheep and buffalo glands is in progress. During these studies a number
of polymorphic forms of PRL were noticed. Some were shown to be charged isomers
while others were shown to differ in degree of sulphation and hence to be size
isomers. The nature of the linkage between polypeptide and sulphur is being
investigated. It could be sugar-SO_4 or tyrosine-SO_4. Further work is in progress.

Acknowledgements

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-association of α-chymotrypsin: Effect of amino acids

T. RAMAKRISHNA and M. W. PANDIT*

Centre for Cellular and Molecular Biology, Hyderabad 500 007, India

Abstract. The concentration-dependent self-association of α-chymotrypsin is known to be influenced by various factors including the presence of small molecules and autolysis products. In this connection the effect of various amino acids on the self-association of α-chymotrypsin has been studied, as a point of interest, by measuring the sedimentation coefficient of α-chymotrypsin. The influence of an amino acid is seen to be governed by the nature of its side chain. Some amino acids do not affect the self-association of α-chymotrypsin at all while some affect it moderately and some others considerably. Functional groups such as the −OH group of Ser or the phenolic ring of Tyr do not seem to influence self-association behaviour. Based on these effects, amino acids could be categorized into 3 groups. Activity studies in the presence of amino acids indicate that the site of self-association and the active-site are probably mutually exclusive.

Keywords. Self-association; α-chymotrypsin; ultracentrifuge; sedimentation coefficient; amino acid effect.

duction

ymotrypsin has been used by several workers as a model system for the study of concentration-dependent self-association of proteins. The self-association of eins is known to be dependent on various factors (Schwert, 1949; Smith and vn, 1952; Rao and Kegeles, 1958; Ackers and Thompson, 1965; Sarfare et al., ; Morimoto and Kegeles, 1967; Tellam and Winzor, 1977; Ikeda et al., 1982c). dit and Rao (1974) showed that the self-association of α-chymotrypsin is enced appreciably by autolysis products present in the solution. It is known that l molecules influence the self-association of proteins considerably (Ikeda et al., a). Certain specific amino acid derivatives have been shown to modify the ciation behaviour of α-chymotrypsin (Ikeda et al., 1982b); however, the effect of us amino acids on the self-association behaviour of α-chymotrypsin has not well studied. This report demonstrates that the presence of amino acids ences the self-association of α-chymotrypsin and that the extent of this effect nds on the nature of the side-chain group(s).

erials and methods

nicals

ce crystallized α-chymotrypsin from Worthington (Batch CD17-JC) or from I Biochemicals (product No. 39009) was used without further purification. no acids were purchased from Calbiochem, USA, E. Merck AG, Germany, or I England. All the chemicals used were of guaranteed reagent grade.

boundaries. All the measurements were made at $25 \pm 1°C$. From pictures taken at different times, the sedimentation coefficients of individual peaks were determined. The data were corrected for temperature and viscosity.

Sedimentation patterns were resolved into individual components from enlarged photographs and relative areas were measured by cutting out the peaks and weighing them. Johnston-Ogston correction was not applied as it is negligible for globular proteins.

Protein concentration was determined spectrophotometrically using a value of 20·6 for $E_{280\,nm}^{1\%}$ (Rao and Kegeles, 1958).

Proteolytic activity

The proteolytic activity of α-chymotrypsin in the presence of amino acids was determined using casein as substrate. Tris–HCl buffer of pH 8·3 was prepared and the ionic strength made up to 0·05 by the addition of KCl. To 1 ml of the Tris buffer containing 5 μg of the enzyme and 1·4 μg of amino acid, 1 ml of 1% casein solution in the same buffer was added. The reaction mixture was incubated at 37°C for 20 min and the reaction stopped by the addition of 3 ml of 5% TCA solution. The precipitate was allowed to settle for 30 min at room temperature and then removed by centrifugation. Proteolytic activity was measured as optical density at 280 nm of the supernatant. The activity of the enzyme in the absence of any amino acid served as control. Change in the activity of the enzyme in the presence of amino acid was calculated with respect to the control.

Results and discussion

The sedimentation patterns of α-chymotrypsin in the presence of amino acids are given in figure 1. All the sedimentation velocity experiments were performed at a concentration of 1·6% of α-chymotrypsin and a concentration of 0·44% of the added amino acid (ratio of amino acid to protein ≃ 1:4). The choice of this ratio was guided mainly by the earlier work of Pandit and Rao (1974) on α-chymotrypsin in which they showed the formation of about 20% autolysis product within a couple of hours. The influence of amino acids on the sedimentation behaviour is quite obvious from the sedimentation patterns. For example, in the presence of Arg, Arg·HCl or Lys, the sedimentation pattern shows only one peak similar to the slow-moving peak for monomers of α-chymotrypsin, indicating that these amino acids influence the self-association behaviour of α-chymotrypsin considerably. Table 1 summarizes the results of the sedimentation velocity experiments. The sedimentation pattern of α-chymotrypsin in the absence of any amino acid consists of an equilibrium reaction boundary with $S_{20,\,w}$ values of 2·94 and 6·06. The slow-moving peak consists of monomers while the fast peak reflects the aggregation reaction in equilibrium controlled by concentration (Pandit and Rao, 1974). Comparison of the $S_{20,\,w}$ values obtained (table 1) indicates that the presence of certain amino acids brings about a
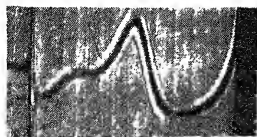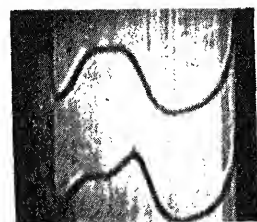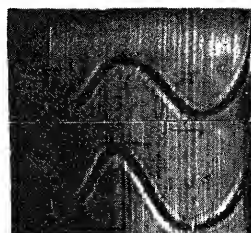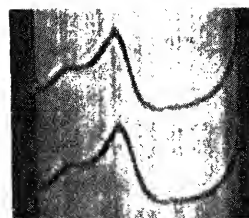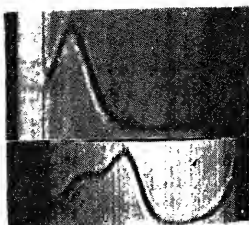
Amino acid added to α-chymotrypsin	S_s (area %)	S_f (area %)	$\bar{S}_{20,w}$	ΔS (%)	Category, based on effect on self-association
Ser	3·27 (22·8)	5·91 (77·2)	5·31	− 2·2	A
Tyr	3·17 (20·6)	5·80 (79·4)	5·26	− 3·1	−''−
Pro	3·13 (26·5)	5·76 (73·5)	5·06	− 6·8	−''−
Ala	3·03 (16·1)	5·51 (83·9)	5·11	− 5·9	−''−
Cys Cys	3·19 (22·4)	5·64 (77·6)	5·09	− 6·3	−''−
Trp	3·18 (22·7)	5·63 (77·3)	5·08	− 6·4	−''−
Phe	2·91 (26·2)	5·67 (73·8)	4·95	− 8·8	−''−
His·HCl	3·16 (48·7)	4·51 (51·3)	3·85	− 29·1	B
Lys·HCl	3·08 (36·0)	4·55 (64·0)	4·02	− 26·0	−''−
Cys	2·77 (35·5)	4·44 (64·5)	3·85	− 29·1	−''−
Glu	3·16 (100)	—	3·16	− 41·8	C
His	2·95 (100)	—	2·95	− 45·7	−''−
Asp	2·98 (100)	—	2·98	− 45·1	−''−
Lys	2·74 (100)	—	2·74	− 49·5	−''−
Arg	2·87 (100)	—	2·87	− 47·1	−''−
Arg·HCl	2·80 (100)	—	2·80	− 48·4	−''−
α-chymotrypsin alone (control)	2·94 (20·3)	6·06 (79·7)	5·43	0	

monomers in the reaction boundary. It is possible to quantify both these influences by computing $\bar{S}_{20,w}$ (the weight-average $S_{20,w}$) from the $S_{20,w}$ values for the individual components and their relative proportions using the formula

$$\bar{S}_{20,w} = (S_s A_s + S_f A_f)/A_t,$$

where S_s and S_f are the sedimentation coefficients ($S_{20,w}$) of the slow-moving and fast-moving components respectively, A_s and A_f are the areas under the respective peaks, A_t is the total area, and $\bar{S}_{20,w}$ is the weight-average sedimentation coefficient for all the species present.

The extent of effect on the self-association could be estimated to a first approximation by computing the per cent difference, ΔS, between the $\bar{S}_{20,w}$ of α-chymotrypsin alone and that in the presence of amino acid. Thus,

$$\Delta S = 100 \left[(\bar{S}_{20,w})_o - (\bar{S}_{20,w})_{+aa} \right]/(\bar{S}_{20,w})_o,$$

where $(\bar{S}_{20,w})_o$ and $(\bar{S}_{20,w})_{+aa}$ are the weight-average sedimentation coefficients of α-chymotrypsin alone and in the presence of amino acid respectively. The $\bar{S}_{20,w}$ values obtained for α-chymotrypsin in the presence of amino acids are given in table 1. The changes in $S_{20,w}$ of individual peaks and their relative proportions obviously influence $\bar{S}_{20,w}$ values in terms of ΔS, as shown in table 1.

By closely examining the values for $\bar{S}_{20,w}$ and ΔS, the amino acids studied could be categorized into 3 groups with respect to their influence on the association behaviour of α-chymotrypsin. The first group of amino acids (category A) affect the sedimentation behaviour of α-chymotrypsin to a negligible extent ($\Delta S \leqslant 9\%$) and the

-moving peak indicating that there is a reduction in the size of aggregates.
.wever, there exist two components of the reaction boundary, though these are
 as well resolved as in the case of category A. The change (ΔS) in the $\bar{S}_{20,w}$ in
 category is in the range 26–29%. The third group of amino acids (category C)
.ws appreciably strong influence on the self-association. The sedimentation
.erns in this case consist of a single peak corresponding to the monomer peak of
.ymotrypsin; ΔS values are in the range 41–50%. It is interesting to see that
.no acids such as His and Lys which greatly affect the self-association of α-
.motrypsin are not as effective in the hydrochloride form as they are in the non-
.rochloride form. It is known that ionic strength of the medium plays an
.ortant role in controlling the extent of aggregation: an increase in the ionic
.ngth normally reduces the extent of aggregation (Pandit and Rao, 1974, 1975).
. change in ionic strength due to the hydrochloride form is expected to be of the
.er of 0·03 leading to an effective ionic strength of 0·08 of the medium. Pandit and
. (1974) have studied the self-association of α-chymotrypsin under conditions of
.lectric pH and 0·05 and 0·1 ionic strength. Comparison of $\bar{S}_{20,w}$ values in table 1
.1 their results indicates that Lys·HCl and His·HCl may influence the self-
.ociation of α-chymotrypsin by increasing the ionic strength. If this contention
. true, then Lys and His should show much lower effect on the self-association
.aviour than their respective hydrochlorides. The change(%) in $\bar{S}_{20,w}$ values
.ught about by the presence of hydrochloride form of amino acid over its non-
.rochloride form can be calculated by using the formula

$$\text{Per cent change} = 100 \left[(\bar{S}_{20,w})_{+aa-\text{HCl}} - (\bar{S}_{20,w})_{+aa}\right]/(\bar{S}_{20,w})_{+aa}.$$

. positive values of the change obtained for Lys$(+46\cdot3\%)$ and His$(+30\cdot5\%)$
.icate that both Lys and His influence the self-association of α-chymotrypsin to a
.ater extent than their hydrochloride forms. Hence, the effect seen in the case of
. HCl and His·HCl cannot be attributed to the increase in ionic strength alone.
.hymotrypsin at ionic strength 0·08 aggregates $(\bar{S}_{20,w}=3\cdot9$; Pandit and Rao,
.5) while in the presence of Arg·HCl it exists in the monomeric form $(\bar{S}_{20,w}=2\cdot8$;
.le 1). Therefore, the reduction in the association brought about by Arg·HCl can-
. be explained merely on the basis of increase in ionic strength.
.he addition of amino acids can influence the self-association of α-chymotrypsin
.ough a change in pH. In the present experiments, under the conditions used the
.nge in pH due to the addition of amino acid was of the order of 0·2 unit in most
.he cases and was 0·4 unit in the case of Lys, Arg and Cys. In the case of Glu and
. pH of the final solution was 4·8 and 4, respectively (change in pH 3·5 and 4·3
.ts, respectively). The reduction in the self-association of α-chymotrypsin in the
.sence of Glu and Asp could thus be attributed to the drastic change in pH. This
. not be true in the case of a majority of the amino acids in category C. These
.lts show that one cannot generalize the effect of amino acids on the self-association
.aviour of α-chymotrypsin. However, whenever there is such an effect its extent
.pears to depend upon the nature of the side-chain of the amino acid.
.t can be seen from the shapes of the patterns and the reduction in the $\bar{S}_{20,w}$ values
.t amino acids having one carboxyl group and one amino (imino in the case of Pro)

category C, which influence the self-association considerably, contain extra carboxyl, amino or imino (imidazole and guanidino) groups in the side-chain. It appears, therefore, that whenever there is a change in the balance of amino and carboxyl groups in added amino acid, the extent of aggregation of α-chymotrypsin is reduced. Cys falls in category B, indicating that the $-SH$ group has an influence on the self-association behaviour of α-chymotrypsin.

Taken together these effects appear to relate to the constellation of charges on α-chymotrypsin involving at least 3 centres. The amino acids may bind to the site of association, if there is any, of α-chymotrypsin, and reduce the self-association by causing complete or partial blocking of the site. This contention is further supported by the fact that the hydrochloride forms of Lys and His had a much smaller effect on the self-association than the non-hydrochloride forms, the effect of the hydrochloride possibly being the neutralization of the influence of one of the charge centres.

It is obvious from these results that at least some of the amino acids, probably through their binding to a specific site on α-chymotrypsin, reduce the extent of self-association. As α-chymotrypsin is a proteolytic enzyme and the process of self-association can be looked upon as a mimicking of substrate–enzyme complex formation (Egan et al., 1957; Kezdy and Bender, 1965), it is likely that the site involved in the self-association is also the active site. Earlier attempts in this direction indicated that the situation is quite complex (Schwert and Kaufman, 1951; Smith and Brown, 1952; Neurath and Dreyer, 1955). Martin and Niemann (1958) investigated the effect of dimerization of α-chymotrypsin on its kinetics and found that the dimer of the enzyme could bind the substrate without hydrolysing it. The studies of Sarfare et al. (1966) on the relationship between the active site and the polymerization site in α-chymotrypsin in the presence of β-phenylpropionate—a competitive inhibitor of the enzyme—indicate that the sites for the polymerization of protein and the binding of the inhibitor are mutually exclusive.

If the active site and the site of association are the same, or if they overlap, either completely or partially, then one would expect that the amino acids that strongly influence the self-association would also reduce the proteolytic activity. In order to find out if this is the case the enzyme was assayed for activity in the presence of various amino acids. The results of these experiments are summarized in table 2. It is

Table 2. Effect of various amino acids on the proteolytic activity of α-chymotrypsin.

Amino acid	Change (%) in proteolytic activity
Ser	+ 12·9
Tyr	+ 16·6
Pro	+ 18·6
Ala	+ 1·0
Cys Cys	+ 5·5
Trp	+ 19·5
Phe	+ 10·0
Lys · HCl	+ 17·4

the other hand, in most cases the presence of amino acid led to a slight increase
0%) in the activity over that in the control. This clearly indicates that the active
of the enzyme is completely free even in the presence of amino acid. It is known
α-chymotrypsin when dissolved in Tris–HCl buffer (pH 8·3 $\mu = 0.05$) undergoes a
-dependent autolysis. Pandit and Rao (1974) showed that most of the autolysis
s place within the first 30 min and the process reaches a steady value of about
. They observed further that the extent of autolysis was decreased to the level of
upon prior addition of autolysis products to the incubation mixture. The
ease in the activity of α-chymotrypsin which we observed in the presence of
no acids may well be the effect of a decrease in autolysis of the enzyme. It is
resting to note that the increase (av. 11%) in the activity of the enzyme in the
ence of amino acids coincides very well with the decrease in autolysis reported by
dit and Rao (1974). Therefore, it is most likely that the increase in activity in the
ence of amino acids is a reflection of reduced autolysis. A number of monomeric
ymes have been found to exhibit either positive or negative co-operativity
unier et al., 1974; Niemeyer et al., 1975; Ainslie and Neet, 1979) when bound by
ctor molecules. Therefore, a second possibility, that the binding of amino acid at
e other site may influence the activity through a conformational change at the
ve site, cannot be ruled out.

conclusion, the presence of amino acids influences the self-association
aviour of α-chymotrypsin. This influence appears to be governed by the nature of
side-chain of the amino acids and is, therefore, related to the charge distribution
ch determines their binding to certain functional groups on the protein. However,
-chains such as the −OH group of Ser or the phenolic ring of Tyr do not
ience the self-association behaviour. The presence of amino acids has a strong
ience on the self-association but does not cause any reduction in the activity,
cating that the active site is not involved in the self-association of α-chymotrypsin
er the conditions studied.

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estimate of the physical distance between two linked markers in emophilus influenzae

E. B. SAMIWALA, VASUDHA P. JOSHI and N. K. NOTANI

Molecular Biology and Agriculture Division, Bhabha Atomic Research Centre, Bombay 400 085, India

Abstract. Using DNA clones, the physical distance between the linked genes *nov* and *str* in *Haemophilus influenzae* was estimated. Although none of the cloned inserts contained both the markers, pJ1-8Str^R 13 (insert of 18·7 kb) included *str* gene at one end and part of *nov* gene at the other end of the insert. By *Eco*RI restriction analysis and by Southern hybridization, the distance between the two *Eco*RI sites, cutting at which inactivates the two genes, was estimated to be 17·7 kb. A single continuous *Eco*RI fragment (containing 4 *Eco*RI sites within it) carrying both the genes intact would need to be 20·4 kb in size. These estimates were confirmed independently using different clones of *nov*^{r} and *str*^{r} alleles as probes for hybridization with *Bam*HI-digested chromosomal DNA.

Keywords. *Haemophilus influenzae*; linked genes *nov* and *str*; physical distance.

oduction

h molecular weight DNA extracted from a *Haemophilus influenzae* strain stant to the antibiotics novobiocin (*nov*^{r}) and streptomycin (*str*^{r}), can co-nsform *H. influenzae* strain sensitive to both antibiotics with a relatively high uency. Although alleles of both these genes have been individually cloned (Setlow *al.*, 1981; Joshi and Notani, 1984; McCarthy and Cox, 1986; Samiwala, 1987; odgal, S. H., unpublished results), the two genes have not been cloned on a single gment. From transformation studies Bagci and Stuy (1979) estimated the physical ance between *nov* and *str* genes to be 15 kb and Joshi *et al.* (1984), using a *nov* e clone, estimated the distance to be greater than 8 kb. The high-efficiency vector -8 (Joshi and Notani, 1983) was used to make a DNA construction which carries gene at one end of the insert and part of *nov*^{r} gene at the other. Using the rmation obtained from the restriction map of pJ1-8Str^R 13 and comparing it with information available from *str*^{r} and *nov*^{r} plasmids, a new, more accurate estimate he physical distance between the two genes was obtained. The present report cribes the construction of the chimeric DNA and the analysis that enabled us to ke the new estimate of the physical distance between *nov* and *str* in *H. influenzae*.

terials and methods

terial strains and plasmids

influenzae strains and plasmids used in the study are listed in table 1.

pJ1-8 plasmid	A DNA cloning vector; resistance to 5 μg/ml (or more) ampicillin; has a single *Eco*RI site outside amp^r marker	Joshi and Notani (1983)
pJ1-8N19 and pJ1-8N2	Chimeric DNA, carries amp^r plasmid marker and nov^r chromosome marker	Joshi and Notani (1984)
pJ1-8Str^R 14	Chimeric DNA, carries amp^r plasmid marker and str^r chromosome marker	E. B. Samiwala and N. K. Notani
pJ1-8Str^R 13	Chimeric plasmid, carries $amp^r str^r$ and part of nov^r markers	Present communication

2 μg/ml NAD (nicotinamide adenine dinucleotide) and 10 μg/ml hemin. For solid medium, 1·2% Difco Bacto agar was added to the broth. Media were sterilized at 15 psi for 18 min. Supplements were added to the medium just before use. Plasmid-bearing cultures were grown in the presence of appropriate antibiotics. Antibiotics were used at the following final concentrations: ampicillin, 5 μg/ml; novobiocin, 2·5 μg/ml; and streptomycin, 200 μg/ml. Strains were preserved by freezing exponential-phase cell cultures at $-73°C$ with 15–20% sterile glycerol.

Extraction of DNA

Chromosomal DNA was isolated according to the method of Marmur (1961). Plasmid DNA was isolated by the method of Hirt (1967), with minor modifications (Notani, 1981). Plasmid DNA was purified by ethidium bromide–cesium chloride (EtBr–CsCl) equilibrium density gradient centrifugation by the method described by Maniatis *et al.* (1982).

Extraction and purification of pJ1-8Str^R 13 DNA

pJ1-8Str^{R}13 DNA was recovered in somewhat low yields. After isolating the plasmid DNA by the usual methods, the cleared lysate was directly mixed with CsCl and EtBr. CsCl–EtBr equilibrium density gradient centrifugation was performed using a Type 65 rotor in a Beckman L8 ultracentrifuge at 139,500 g for 60 h at 20°C. At the end of the run, the tubes showed only one band, which on investigation proved to be the chromosomal DNA band. Solution from the region below the chromosomal band (where plasmid DNA is expected to band) was collected from 6 tubes and pooled together into one tube. Equilibrium density gradient centrifugation was carried out once again. This time a faint lower band was obtained, which was recovered. This consisted of the covalently closed circular (CCC) form pJ1-8Str^{R}13. This was used for all experiments.

^{32}P-Labelling of plasmid DNA

^{32}P-Labelled plasmid DNA was made by the method of Kahn *et al.* (1983).

All restriction enzymes and T4 ligase were purchased from Bethesda Research Laboratories and used according to the instructions provided.

Southern hybridizations

Southern hybridization was carried out as described by Maniatis *et al.* (1982).

Genetic transformation

The transformation mixture consisted of 0·8 ml BHI + 0·1 ml DNA (> 1 μg) + 0·1 ml competent cells (cells made competent by the method of Goodgal and Herriott, 1961). The mixture was incubated for 10–15 min at 37°C for uptake of DNA. Appropriate dilutions were then made in saline and cells were pour-plated with 10 ml BHI agar. After incubation at 37°C for 1·5–2 h, BHI agar (10 ml) containing the appropriate antibiotic(s) was added to the plates.

Results and discussion

Construction of pJ1-8Str^{R}13

Linkage of *nov* and *str* genes in *H. influenzae* is known (Voll and Goodgal, 1961). An attempt was made to clone the entire segment of chromosomal DNA which carries both *nov* and *str* genes. For this purpose, *nov*^{r}*str*^{r} chromosomal DNA was digested with *Eco*RI for a very short time (1 μg DNA was digested with 1 unit *Eco*RI for 5 min in 20 μl volume) because it is known that *Eco*RI inactivates both *nov*^{r} and *str*^{r} genes as well as breaks the linkage between the two genes (Samiwala, 1987). Inactivation of *nov*^{r} and *str*^{r} marker transformation activity by the treatment used was only 13 and 28% respectively (data not shown). This DNA was ligated to *Eco* RI-digested pJ1-8 DNA (which carries the *amp*^{r} marker). Transformation of competent wild-type Rd cells with ligated DNA failed to yield any Nov^{R}Str^{R}Amp^R colonies but one of the Str^{R}Amp^R colonies was found, by hybridization with the pJ1-8N 19 probe, to be carrying part of *nov*^{r} allele along with the entire intervening sequence and the *str*^{r} gene on the plasmid. This plasmid was designated pJ1-8Str^R 13.

Determination of the size of plasmid pJ1-8Str^R 13

Restriction analysis of this plasmid was carried out using ^{32}P-labelled DNA. ^{32}P-Labelled pJ1-8Str^{R}13 DNA was digested with *Eco*RI and electrophoresed on agarose gels. Autoradiography of the gels revealed that pJ1-8Str^{R}13 consists of 5 *Eco*RI fragments (figure 1). Two of the fragments are the 2·2 kb and 1 kb fragments which carry the *str*^{r} allele. The other 3 are 9·8, 5·7 and 3·8 kb fragments. When a

9.8

5.7

3.8

2.2

(a)

(b)

9.8

5.7

3.8

1.7

1.0

(2)
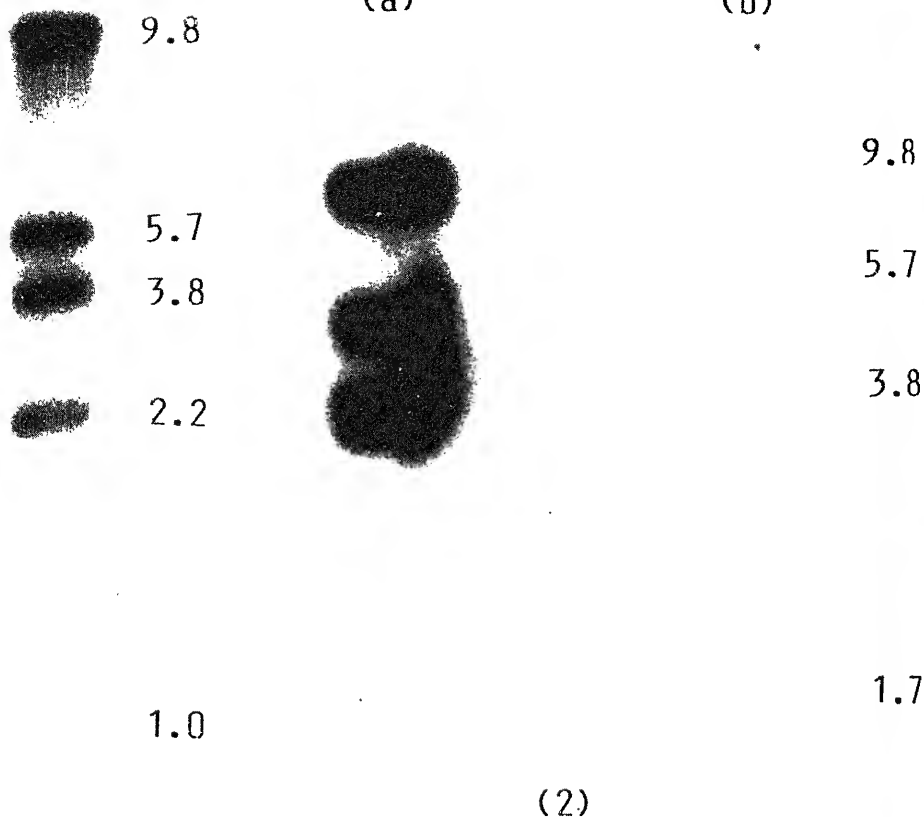
(1)

Figures 1 and 2. 1. Restriction pattern obtained by digesting ^{32}P-labelled pJ1-8Str^{R}13 DNA with EcoRI. Numbers indicate DNA fragment size in kb. 2. Southern hybridization of EcoRI digested pJ1-8N19 (lane a) and pJ1-8Str^{R}13 (lane b) DNAs with ^{32}P-labelled pJ1-8N19 probe. The 1·7 kb EcoRI fragment is missing in the case of pJ1-8Str^{R}13. Numbers indicate DNA fragment size in kb.

(figure 2). Since pJ1-8Str^{R}13 does not carry the 1·7 kb EcoRI fragment which carries the rest of the nov gene (Joshi and Notani, 1984), it fails to transform cells to the Novr phenotype. Based on these data, a physical and genetic map of the pJ1-8Str^{R}13 insert was prepared and compared with those of the pKLT1 (McCarthy and Cox, 1986), pJ1-8Str^{R}14, pJ1-8N19 and pJ1-8N2 inserts (figure 3). The size of pJ1-8Str^{R}13 was estimated to be 22·5 kb. The physical distance between the two EcoRI sites, cutting at which inactivates the nov^r and str^r genes, is 17·7 kb. The physical distance between the intact nov^r and str^r genes is estimated to be less than 20·4 kb.

Confirmation of the physical distance between the two genes
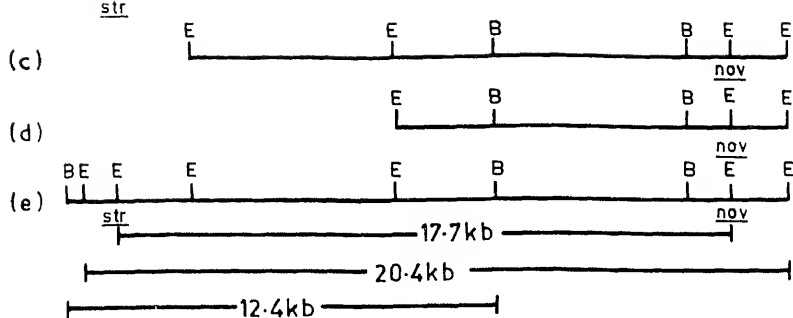
Figure 3. Restriction and genetic maps of chromosomal DNA inserts of (a) pJ1-8Str^{R}14; (b) pJ1-8Str^{R}13; (c) pJ1-8N19 and (d) pJ1-8N2. (e) Chromosomal map deduced from the information available from (a) to (d) and McCarthy and Cox (1986). E, EcoRI site; B, BamHI site.
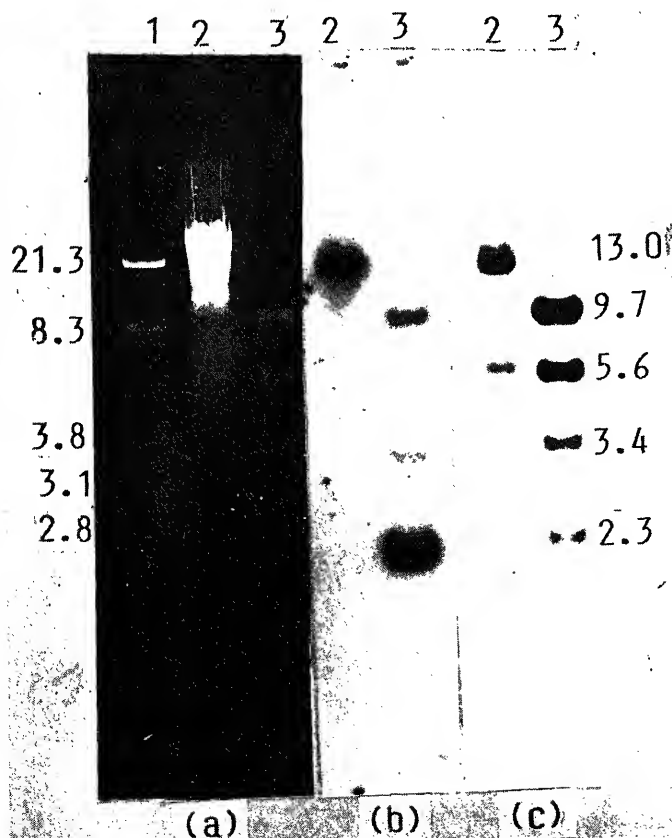
Figure 4. (a) Agarose gel (1%) electrophoresis of BamHI digested H. influenzae chromosome (lane 2) and pJ1-8N19 (lane 3), and HpaI-digested T7 DNA as standard (lane 1). Numbers indicate DNA fragment size in kb. (b) Southern blot of gel in (a) probed with nick-translated pJ1-8Str^{R}14 DNA. (c) Southern blot of gel in (a) probed with nick-

McCarthy and Cox (1986), a *Bam*HI site lies to the left of *str* gene (0·6 kb away from the left-most *Eco*RI site, figure 3a). Another *Bam*HI site is on the 9·8 kb *Eco*RI fragment (figure 3b) which carries part of the *nov* gene. The *Bam*HI fragment is thus 12·4 kb in size. If this estimate is correct, then *Bam*HI-digested chromosomal DNA should show a fragment which hybridize with both *nov*^{r} (pJ1-8N19) and *str*^{r} (pJ1-8str^{R}14) probes and this fragment should be 12–13 kb in size. This was indeed found to be true (figure 4): the *Bam*HI fragment which hybridizes with both *nov*^{r} and *str*^{r} probes was found to be 13 kb in size. This estimate is somewhat higher than the estimates made earlier. The estimate of Joshi *et al.* (1984) of greater than 8 kb was constrained by the length of the *nov*^{r} alone available at that time. Bagci and Stuy's (1979) estimate may have limitations in the use of uncloned DNA for transformation. However, the latter is underestimated only by about 5 kb. Using uncloned DNA, an average size of the insert (2 crossovers) during transformation was estimated at about 9 kb (Notani and Goodgal, 1966). Thus, if exchanges were purely random and without interference, roughly 4 exchanges would occur between *nov* and *str* genes.

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enzyme-linked immunosorbent assay using the avidin–biotin for detection of circulating antigen in bancroftian filariasis

K. A. PARKHE, P. RAMAPRASAD and B. C. HARINATH*

Mahatma Gandhi Institute of Medical Sciences, Sevagram 442 102, India

Abstract. Detection of filarial antigen in different groups of sera was carried out by sandwich as well as inhibition enzyme-linked immunosorbent assays using antibody-coated sticks. Both systems were found to be equally sensitive in detecting antigen in 90% of microfilariae carriers. Incorporation of avidin–biotin in the sandwich assay system increased the sensitivity of antigen detection from 10^{-6} to 10^{-16} pg. A 67% decrease in the number of false negative results was observed when the sensitive avidin–biotin inhibition enzyme-linked immunosorbent assay system was used for analysis of filaria blood samples.

Keywords. *Wuchereria bancrofti*; filarial serum immunoglobulin G; enzyme-linked immunosorbent assay; biotinylated FSIgG; biotinylated ES antigen; avidin penicillinase.

ction

stic methods based on detection of parasite antigens are more useful in
g active infection. Filarial antigens have been detected in blood, urine and
ele fluid samples of filarial patients and animals (Kaliraj *et al.*, 1979;
ayake *et al.*, 1982, 1984; Hamilton *et al.*, 1984; Reddy *et al.*, 1984, 1986;
ra *et al.*, 1985a, b).

lose acetate membranes (CAM) attached to plastic strips have been used in
-linked immunosorbent assays (ELISA) for the detection of filarial antibody
et al., 1986). This paper reports the use of CAM attached to plastic strip
LISA) for detection of antigen in sandwich as well as inhibition ELISA.

strong interaction between avidin and biotin has been utilized in various
such as specific staining of biological membranes in electron microscopy
ann and Richards, 1974), selective absorption of cells (Jasiewicz *et al.*, 1976),
benzymatic techniques (Guesdon *et al.*, 1979), and competitive inhibition
Wilson *et al.*, 1986) in the detection of circulating antigen levels in mice
with *Toxocara canis* using direct ELISA (Bowman *et al.*, 1987). This
nication reports the adaptation of stick ELISA to the detection of circulating
in filaria blood samples, and the increased sensitivity achieved by using the
biotin system.

Materials and methods

Sera

Human sera (30 samples), belonging to different groups, from normal subjects (non-endemic and endemic normal) and filarial patients (microfilaraemic and clinical filariasis) were screened. Serum was separated and stored at $-20°C$ after addition of sodium azide (0·1%) as preservative.

Filter paper blood samples were collected as described previously (Malhotra *et al.*, 1982).

Wuchereria bancrofti microfilariae excretory–secretory antigens

Wuchereria bancrofti microfilariae excretory–secretory antigen (*Wb* mf ES Ag) was prepared as described previously (Kharat *et al.*, 1982). The culture fluid was centrifuged at 13,000 *g* for 15 min. The supernatant (25 ml aliquots) was dialysed and lyophilized. The lyophilized powder was reconstituted in 2 ml of 0·05 M sodium phosphate buffer (SPB), pH 7·2 and protein was determined according to the method of Lowry *et al.* (1951).

Immunoglobulin-G fraction of human filarial serum immunoglobulins

The immunoglobulin G fraction of human filarial serum immunoglobulins (FSIgG) was prepared as described by Reddy *et al.* (1984).

Sandwich and inhibition ELISA

Twenty mg of FSIgG were conjugated to 1000 units of penicillinase (Sigma Chemical Co., USA) by the method of Avrameas (1969).

Conjugation of *Wb* mf ES Ag and penicillinase was achieved as described for the FSIgG–penicillinase conjugate, except that 125 μg of ES Ag protein was used instead of 20 mg. The substrate consisted of soluble starch (150 mg) in 27·5 ml of SPB (pH 7·2, 0·25 M) containing 10·6 mg of penicillin 'V' and 100 μl of 0·08 M iodine in 3·2 M potassium iodide solution. The substrate was prepared fresh before use.

Stick ELISA was carried out in small plastic vials (9×55 mm). Optimum amounts of FSIgG (in 5 μl SPB, pH 7·2, 0·05 M) containing 100 ng of protein, sera (1:600 dilution in PBS/T), FSIgG penicillinase conjugate (1:200 dilution in PBS/T), ES antigen penicillinase conjugate (1:100 dilution in PBS/T) were determined by Chequer Board titration.

Sandwich ELISA was carried out as described by Reddy *et al.* (1984) with some modifications. After applying 5 μl of FSIgG on CAM, the sticks were dried at room temperature and incubated at 37°C for 2 h with 3% gelatin (diluted in SPB, pH 7·2,

...ve result.

...ibition ELISA was carried out as described by Malhotra et al. (1984) and
...prasad et al. (1985). The procedure is the same as described above for sandwich
...A except that 0·5 ml of ES Ag–penicillinase conjugate was added instead of
... of FSIgG–penicillinase conjugate. A positive reaction in inhibition ELISA was
...ted by the persistence of blue colour.

labelling of FSIgG and mf ES Ag

...droxysuccinimidobiotin (Sigma Chemical Co., USA) was conjugated to FSIgG
...scribed by Guesdon et al. (1979) and Heitzmann and Richards (1974) with some
...ications. A solution of 10 mg FSIgG in 1 ml of 0·1 M $NaHCO_3$ was mixed with
... of N-hydroxysuccinimidobiotin solution (12 mg in 1 ml of dimethyl
...amide). The mixture was kept at 25°C for 1 h and then dialysed at 4°C against 5
...ges of SPB (pH 7·2, 0·01 M). After dialysis, the conjugate was removed and kept
...20°C with 0·02% sodium azide as a preservative until used.

...tin labelling of Wb mf ES Ag was carried out in the same way except that
...g of Wb mf ES Ag protein was used instead of 10 mg FSIgG.

ling of avidin to penicillinase

...in was coupled to penicillinase as described by Guesdon et al. (1979). To 1 ml of
...(pH 7·2, 0·25 M) containing 100 units of penicillinase and 2 mg of avidin (Sigma
...nical Co., USA) was added 40 μl of a 1% aqueous solution of glutaraldehyde,
...stirring. After 3 h at 25°C, the preparation was dialysed for 48 h against 7
...ges of SPB (pH 7·2, 0·01 M) at 4°C. It was then centrifuged at 4°C (30 min at
...g) and kept at −20°C after addition of 0·02% sodium azide as a preservative
...used.

wich and inhibition ELISA with avidin–biotin

...optimum dilutions of FSIgG (5 μl containing 100 ng protein), filter paper blood
...les (1:600,000 dilution in PBS/T), biotinylated FSIgG (1:1000 dilution in
.../T), biotinylated ES Ag (1:1000 dilution in PBS/T) and avidin–penicillinase
...ugate (1:2000 dilution in PBS/T) were determined by Chequer Board titration.
...ndwich ELISA with avidin–biotin was carried out as follows. After applying 5 μl
...SIgG on CAM, sticks were dried and incubated at 37°C for 2 h with 3% gelatin.
...r washing, sticks were incubated at 37°C for 2 h with different dilutions of Wb mf
...Ag, viz., 120, 12, 1·2, 0·12, 10^{-3}, 10^{-6}, 10^{-7}, 10^{-10}, 10^{-13}, 10^{-16}, 10^{-17}, 10^{-18}
...10^{-19} pg/ml; PBS/T was used for the control. The sticks were washed and
...bated at 37°C for 2 h with 0·5 ml of biotinylated FSIgG. After washing, the sticks
...e incubated at 37°C for 2 h with 0·5 ml of avidin–penicillinase conjugate. After a
...ough wash (10 times) with PBS/T, 0·5 ml of freshly prepared substrate was

was the same except that 0·5 ml of biotinylated ES Ag–conjugate was used instead of biotinylated FSIgG and filter paper blood eluates were used for antigen detection instead of *Wb* mf ES Ag. The persistence of blue colour denoted positive reaction.

Results

A total number of 30 sera belonging to different groups were screened by the stick ELISA method. Nine out of 10 microfilaraemic sera, 8 out of 10 clinical filariasis sera and none of the 5 non-endemic and 5 endemic normal sera showed the presence of antigen by sandwich ELISA. In inhibition ELISA, 9 out of 10 microfilarial sera, 6 out of 10 clinical filariasis sera and none of the non-endemic and endemic normal sera showed the presence of antigen.

To determine the detectable limits of antigen by sandwich ELISA with and without the avidin–biotin system, different dilutions of *Wb* mf ES antigen were used, from 120 pg/ml up to 10^{-19} pg/ml. Antigen at concentration as low as 10^{-6} pg/ml was detected by sandwich ELISA; with incorporation of avidin–biotin the assay could detect as little as 10^{-16} pg/ml antigen. As inhibition ELISA detects specific antigen, further studies were carried out by inhibition ELISA. Filter paper blood eluates were used in place of sera, as described earlier (Malhotra and Harinath, 1984).

Twenty-two out of 191 microfilaraemia samples in the form of filter paper blood eluates were antigen-negative while the remaining were antigen-positive by inhibition ELISA in PVC plate assay (Ramaprasad, P., Bharati, M. S. and Harinath, B. C., unpublished results). Fifteen of these 22 antigen-negative filter paper blood eluates and 5 out of the remaining 169 antigen-positive samples were rescreened in inhibition ELISA incorporating the avidin–biotin system. Ten of these 15 "antigen-negative" microfilaraemia samples and all the 5 antigen-positive samples showed the presence of antigen.

Discussion

The diagnosis of filariasis based on the detection of antifilarial antibody has been extensively explored. However, detection of parasite antigen in body fluids may be more informative than antibody detection in the confirmation of active infection (microfilaraemia). The presence of circulating antigen has been reported in 77 and 82% of microfilaraemia carriers using counter-immunoelectrophoresis and sandwich ELISA respectively in bancroftian filariasis (Kaliraj et al., 1981; Reddy et al., 1984). Microfilariae ES antigen has also been determined in 70–75% of microfilaraemics by inhibition ELISA (Malhotra and Harinath, 1984; Ramaprasad et al., 1985). Rabbit antiserum raised against bovine serum albumin was used in dilutions ranging from 1:4000 to 1:512,000 in the avidin–biotin technique (Guesdon et al., 1979).

Stick ELISA (indirect), which was developed earlier (Parkhe et al., 1986) for detection of antibody, has been adapted for detecting antigen. Further, incorporation of avidin–biotin in stick ELISA was attempted with a view to increase the sensitivity of detection of circulating antigen by sandwich as well as inhibition ELISA. The use of avidin–biotin in sandwich ELISA enhanced the sensitivity of the assay; the

ng circulating antigen.

wledgement

ork was supported in part by the Indo-US S and T Initiative Programme and
Department of Biotechnology.

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unol. Methods*, **87**, 59.

t of altered sterol levels on the transport of amino acids and rane structure of *Microsporum gypseum*

SEEMA VAIDYA, GEETA BHARTI, RAKESH PANDEY and
G. K. KHULLER*

Department of Biochemistry, Postgraduate Institute of Medical Education and Research,
Chandigarh 160 012, India

Abstrat. Ergosterol and cholesterol supplementation resulted in a significant increase (1·5-fold) in the sterol content while phospholipid remained unaffected in *Microsporum gypseum*. The levels of phosphatidylethanolamine and phosphatidylcholine increased in ergosterol supplemented cells. However, a decrease in phosphatidylcholine and an increase in phosphatidylethanolamine was observed in cholesterol grown cells. The ratio of unsaturated to saturated fatty acids decreased on ergosterol/cholesterol supplementation. The uptake of amino acids (lysine, glycine and aspartic acid) decreased in sterol supplemented cells. Studies with fluorescent probe 1-anilinonaphthalene-8-sulfonate showed structural changes in membrane organisation as evident by increased number of binding sites in such cells.

Keywords. Ergosterol; cholesterol; phospholipids; fatty acids; amino acids; transport; *Microsporum gypseum*.

iction

, one of the essential components of eukaryotes impart the mechanical strength membrane due to their ordered packing in phospholipid bilayer which is al to maintain the structural integrity of the cell (Park, 1978). These nents control the permeability of the membrane in conjuction with acyl of phospholipids (Bloch, 1983; Prasad, 1985). It has been demonstrated that influence membrane function such as passive transport, carrier mediated ort and enzymatic activity of membrane bound enzymes (Demel and Kruyff, Polyene antibiotics have been extensively used to study the importance of in fungal membranes as membrane sterols are known to interact with them eb *et al.*, 1958; Lampen *et al.*, 1960). Lipid metabolism and influence of nolipid polar head groups and fatty acyl chains on the permeability properties nbranes in dermatophytes have been studied (Larroya and Khuller, 1985, 1986; y *et al.*, 1987), whereas no information is available on the role of sterol on ibility behaviour of membranes of fungi except *Candida albicans* and *Aspergillus* Singh *et al.*, 1979a, b; Mazumdar *et al.*, 1987). In this study *Microsporum* m, a dermatophyte, was grown in the medium supplemented with ergosterol (a l sterol) and cholesterol (a sterol foreign to fungus), in order to examine the nduced changes in membrane lipid composition and its impact on the structure nction of membrane of this fungus.

Radiolabelled [U-^{14}C]-L-aspartic acid, [U-^{14}C]-L-lysine and [U-^{14}C]-glycine were procured from Bhabha Atomic Research Centre, Bombay. Cholesterol, ergosterol, L-lysine, glycine and L-aspartic acid were obtained from Sigma Chemical Co., St. Louis, Missouri, USA, Novozyme, 234, was procured from Novo-industries, Baegsvaerd, Denmark. Cellulase 'CP' was obtained from John and E. Sturge, North Yorkshire England. 1-Anilinonaphthalene-8-sulfonate (ANS) was a product of Fluka, Switzerland.

Growth of culture

M. gypseum, obtained from the Mycological Reference Laboratory, School of Hygiene and Tropical Medicine, London was grown as shaking cultures in Sabouraud's broth (4% glucose, 1% peptone, pH 5·4–5·6) at 27°C. Varied concentrations of ergosterol/cholesterol in ethanol (0·1%) were added to the growth medium before inoculation. Cells were harvested in the mid log phase (4 days) and processed further as per the following methods.

Quantitation of lipids

Lipids were extracted by the method of Folch *et al.* (1957). Phospholipids were quantitated by the method of Marinetti (1962). Individual phospholipids were separated by single dimensional thin-layer chromatography in chloroform:methanol: 7N ammonia (65:25:4, v/v). Methyl esters of phospholipid fatty acids (separated from the neutral lipids by acetone precipitation) were prepared by transesterification with methanol in the presence of thionyl chloride (Khuller *et al.*, 1981) and were resolved on a Perkin Elmer gas liquid chromatograph fitted with a 0 V-225 column at 190°C using nitrogen as a carrier gas (the flow rate was 40 ml/min). Fatty acid methylesters were identified by comparison of their retention times with that of authentic standards. Fatty acids were quantitated by triangulation of peak area.

Quantitation of sterols

Sterols were extracted by boiling the cells in alcoholic KOH for 1 h (Singh *et al.*, 1979).The hydrolysates were cooled and extracted thrice with petroleum ether and quantitated by the method of Zlatkis *et al.* (1953).

Uptake studies of amino acids

This was examined by incubating 1 ml of cell suspension (40–50 mg fresh weight of cells in 1 ml of citrate phosphate buffer, pH 6·5) at 27°C for different time periods. The reaction was initiated by addition of labelled amino acid 5 mM (specific acitivity 120 μCi/mmol) and was stopped by diluting it with chilled normal saline and filtering through 0·45 μM millipore membrane filters. After washing 2–3 times with chilled normal saline (0·85% NaCl) filters were dried, weighed and counted in toluene based

roplasts were prepared according to the method of Larroya *et al.* (1984).
res were incubated under sterile conditions with 30 mg of each Novozym '234'
ellulase 'CP' in 10 mM citrate-phosphate buffer (pH 6·5) containing 0·7 M NaCl
8 h at 30°C. Formation of spheroplasts was monitored microscopically. The
ation mixture was centrifuged at 1000 *g* for 10 min and the supernatant
ining the cell wall degrading enzymes was discarded. Pellet was washed twice
the buffer and the spheroplasts were purified by centrifugation on a Ficoll-
e gradient at 400 g for 15 min. The purified spheroplasts free of cell debris
ered from the supernatant were used for further studies.

tural studies

rescent probe, ANS was used for structural studies. The basic assay mixture
sted of a total volume of 2 ml in 10 mM citrate phosphate buffer, pH 6·5
ining 0·7 M sodium chloride, 10 μM ANS and spheroplast protein ranging
50–200 μg. The fluorescence emission was recorded on a Kontron SFM-25
rofluorimeter. The number of binding sites were calculated from Scatchard plot
sis (Azzi, 1974). Spheroplast protein was estimated by the method of Lowry
(1951). The statistical significance of the results was tested by Student's *t* test.

ts and discussion

ges in the levels of sterols are likely to alter the structural and functional aspects
embranes. In this study, *M. gypseum* was grown in the medium supplemented
different concentrations of cholesterol and ergosterol. Optimum concentrations
olesterol and ergosterol were determined by measuring the levels of total sterols
phospholipids in supplemented cells and the concentration which induced
mum alteration in sterol levels with marginal changes in phospholipids was
ed. Figure 1 shows that 50 and 80 μg/ml of ergosterol and cholesterol, respec-

these concentrations. The optimum concentration determined for cholesterol was much higher as compared to ergosterol which is probably due to the different behaviour of ergosterol and cholesterol. In an earlier study, Pinto *et al.* (1985), have reported that ergosterol is taken up by *Saccharomyces cerevisiae* cells up to a certain concentration of exogenous sterol, while cholesterol content plateau at a much higher concentration of sterol in the medium. In addition, uptake of sterol from the medium through the plasma membrane and cell wall need not be equivalent for all sterols as ergosterol partitions through phospholipid bilayer with great difficulty in comparison with cholesterol (Park, 1978). Both these sterols at optimum concentration had a stimulatory effect on growth of *M. gypseum* which is unexplainable. However, the stimulatory effect was more with cholesterol which is similar to the observation of Wright *et al.* (1983), where ergosterol when added alone or in combination with fatty acids had a slight but consistant stimulatory effect on the growth of *Talaromyces thermophilus*.
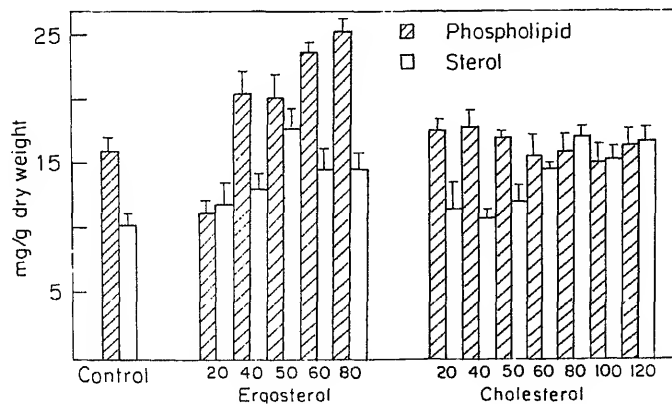
Total sterols increased (1·5-fold) significantly on supplementation of ergosterol and cholesterol while no change was seen in total phospholipids (table 1). These results indicate the capacity of *M. gypseum* to take up sterols from the medium. Alterations were also observed in the individual phospholipid composition (table 2).

Table 1. Effect of sterol supplementation on phospholipid and sterol content of *M. gypseum*.

	Total phospholipids	Total sterol
	mg/g dry wt.	
Control	16·14 ± 1·21	10·35 ± 1·39
Cholesterol[a]	16·30 ± 1·49[NS]	16·69 ± 0·83[b]
Ergosterol[a]	20·48 ± 2·48[NS]	17·79 ± 1·78[a]

Values are mean ± SD of 4 independent batches.
NS, Not significant.
[a]Optimum concentrations (50 and 80 μg/ml of ergosterol and cholesterol, respectively were used).
[b]$P \leqslant 0.01$.

Table 2. Effect of sterol supplementation on individual phospholipid composition of *M. gypseum*.

Phospholipid fraction	Control	Ergosterol[a]	Cholesterol[a]
		(mg phospholipids/g dry wt.)	
LPC	2·83 ± 0·15	2·89 ± 0·18[NS]	3·24 ± 0·73[NS]
PS + PI	4·28 ± 0·82	4·5 ± 0·38[NS]	3·76 ± 0·55[NS]
PC	6·23 ± 0·12	8·15 ± 0·90[b]	5·03 ± 0·36[c]
PE	2·22 ± 0·35	3·70 ± 0·56[b]	3·22 ± 0·46[b]
Unknown PL	1·15 ± 0·07	0·38 ± 0·05[c]	1·07 ± 0·09[NS]
LPC + PC + PE/PS + PI	2·64	3·28	3·05

Values are mean ± SD of 4 independent batches. NS, Not significant.

ethylation of PE to PC in yeast mutant GL 7. On the other hand, a decrease in
ɑnd an increase in PE levels were observed on cholesterol supplementation.
 supplementation also induced changes in the membrane surface charge as
ʋed by alterations in ratio of zwitterionic to anionic phospholipids. Alterations
ʋed in individual phospholipid components as well as their acyl group com-
on indicate the capacity of the cell to adjust its phospholipid composition in
ɑ manner so as to maintain the normal functioning of the cell. Phospholipid
ᴄid composition did not alter on sterol supplementation. However, a significant
ɪse in the ratio of unsaturated to saturated fatty acids was seen (table 3), which
ɴainly accomplished by increased amount of palmitic acid (35–43%) with a
ɪse in linoleate (18:2) in sterol supplemented cells as compared to control cells.
ɾease in the ratio of unsaturated/saturated fatty acids on supplementation of
ʂ indicates a rigidifying effect of sterols on the membrane. Buttke et al. (1980)
ɓserved invariably high percentage (40–45%) of palmitic acid and stearic acid
ʜ the major phospholipids, PC and PE with ergosterol as the sterol source as
ɑred to the cells supplied with 7-dehydrocholesterol in yeast.

Table 3. Effect of sterol supplementation at optimum concentration on phospholipid fatty acid composition of *M. gypseum*.

Fatty acids	Control	Ergosterol (relative percentage)	Cholesterol
$C_{12:0}$	2·313	2·015	2·588
$C_{14:0}$	1·813	2·506	2·638
$C_{16:0}$	21·807	29·525	31·207
$C_{16:1}$	2·648	1·545	1·152
$C_{18:0}$	2·763	2·819	0·852
$C_{18:1}$	32·630	28·387	31·498
$C_{18:2}$	36·020	33·201	30·063
U/S	2·485	1·712	1·682

Values of average of two independent runs.

 lipid composition of cell membrane plays an important role in regulating the
ons of cells. Therefore permeability properties of intact cells were examined by
ɔring the uptake of amino acids (lysine, glycine and aspartic acid) for different
 periods. Maximum uptake was seen after 5 min of incubation, hence the
ɓort studies were carried out at this time point. A significant reduction in the
ᴇ of lysine, glycine and aspartic acid was observed in both cholesterol and
ᴛerol supplemented cells as compared to control cells (table 4). Elevated levels of
ʂ and increased saturation of phospholipid fatty acids (tables 1 and 3) are
ɓly responsible for decreased amino acid uptake as these components are
ɴ to reduce membrane permeability (Demel et al., 1972; Prasad, 1985). Singh
 (1979) also reported a significant reduction in the uptake of lysine, glycine,
ᴇ and glutamic acid in *Candida albican* enriched with ergosterol when grown
ᴅroquinone supplemented medium.

Table 4. Uptake of amino acids by *M. gypseum* cells grown in the presence of choles... and ergosterol.

| | | Glycine | |
	Lysine	(n mol/100 mg dry wt./5 min)	Aspartic aci...
Control	$8751 \pm 120\cdot8$	$8002 \pm 128\cdot0$	$1502 \pm 125\cdot6$
Ergosterol[a]	$7752 \pm 135\cdot0^{b}$	$4750 \pm 130\cdot8^{c}$	$1374 \pm 130\cdot4$
Cholesterol[a]	$6750 \pm 105\cdot2^{c}$	$6003 \pm 135\cdot8^{c}$	$1173 \pm 121\cdot2$

Values are mean $\pm$ SD of 3 independent batches.

NS, Not significant.

[a]Optimum concentrations (50 and 80 μg/ml of ergosterol and cholesterol, respect... were used).

[b]$P \leqslant 0\cdot05$; [c]$P \leqslant 0\cdot01$.

Structural changes occurring due to the alterations in the membrane lipid c... position were examined using ANS, a membrane probe. ANS binds non-covale... to proteins and lipids of the membrane and its binding to the phospholipids is in... region of their polar headgroups. Hence it can provide information regar... membrane surface charge and the microenvironment of the bound dye (Azzi *et*... 1969; Brocklehurt *et al.*, 1978). The number of binding sites, as calculated from... Scatchard plots showed a significant increase in spheroplasts prepared f... ergosterol and cholesterol supplemented cells (figure 2). Though the numbe... binding sites for proteins are less than those for the lipids, yet due to their very... affinity for binding to ANS, the protein binding sites remain saturated at... concentration of dye. Therefore increasing the dye concentration affects lipid bin... sites more efficiently, hence changes due to alteration of phospholipid composi... can be studied with ANS (Zierler and Rogus, 1978). Increased binding sites migh... due to changes in the membrane surface charge resulting from changed rati... zwitterionic to anionic phospholipids as suggested by Au *et al.* (1986) as wel... altered ratio of unsaturated/saturated fatty acids of phospholipids as seen f... tables 2 and 3. The changes observed in ANS binding may also be due to chan... hydrophobic environment around the embedded dye, due to integral membr... proteins (Slavik, 1982). Since the dye binds to the membrane lipid and prote...
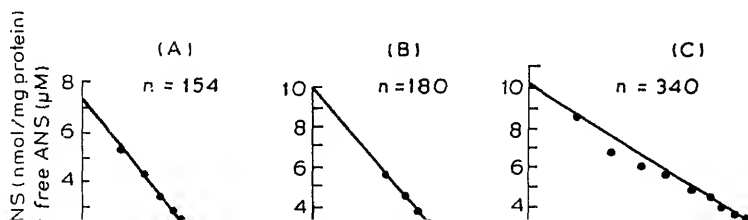
ANS binding and indicate structural and conformational changes in the membrane.

In brief the results of this study suggest that sterols alongwith phospholipid fatty acids have a role in regulating the permeability of the cell as well as the structural aspects of membrane in *M. gypseum*.

Acknowledgement

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MONIKA SAREEN and G. K. KHULLER*

Department of Biochemistry, Postgraduate Institute of Medical Education and Research, Chandigarh 160 012, India

Abstract. The composition, subcellular distribution and rate of synthesis of phospholipids were compared in ethambutol susceptible and resistant strains of *Mycobacterium smegmatis*. Significant quantitative alterations in phospholipids accompanied the acquisition of resistance, whereas fatty acyl group composition of total phospholipid remained the same in ethambutol resistant and susceptible strains. Cell wall of resistant strain exhibited an accumulation of phospholipids and a decrease in the degree of unsaturation of phospholipid fatty acyl groups. Changes in the cell wall phospholipid composition may contribute to resistance of *Mycobacterium smegmatis* to ethambutol.

Keywords. *Mycobacterium smegmatis*; phospholipids; fatty acids; ethambutol; susceptible; resistant; cell membrane; cell wall.

...uction

...ibutol (EMB) is an effective and specific antitubercular drug which is ...ntly used in combination with other antitubercular drugs in chemotherapeutic ...ens (Iseman and Goble, 1988). Previous reports in literature have dealt with the ...of EMB on nucleic acid metabolism (Forbes *et al.*, 1965; Bacalao and Reiber, ...mycolic acid synthesis (Takayama *et al.*, 1979; Kilburn and Takayama, 1981) ...hospholipid metabolism in mycobacterial species (Kilburn *et al.*, 1981; Cheema ...Khuller, 1985). Kilburn *et al.* (1981) observed a decrease in phospholipid ...sis and leakage of phosphatidylethanolamine (PE) out of the cells in ...*bacterium smegmatis*, upon EMB exposure.

...osphospholipids are the major constituents which are associated with the transport ...tabolites across the membrane (Fourcans and Jain, 1974). In general anti-...bial action of the drugs involve transport of the drug from external environ-...to some site on or with in the cell followed by certain alterations in cell ...rane (Beggs and Andrews, 1973). There are several reports which suggest that ...organisms alter their lipid composition in order to resist the toxic effect of ...(Suling and O'Leary, 1977; Gilleland *et al.*, 1984; David and Rastogi, 1985). In ...bacteria, a genus with a high percentage of lipids in cell envelope, investigations ...ing analysis of phospholipid metabolism of drug resistant strains have not ...carried out. Hence, the present investigation was carried out to compare the ...holipid composition, distribution and metabolism in EMB-susceptible and ...nt strains of *M. smegmatis* ATCC 607.

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[1-14C]-Sodium acetate obtained from Bhaba Atomic Research Centre, Bombø and EMB procured from Lederl Laboratories, USA were used.

Bacterial strain and cultivation

M. smegmatis ATCC 607, originally obtained from NCTC, London was use Resistant mutant of M. smegmatis was isolated by replica plating technique described by Lederberg and Lederberg (1952). Stability of the mutants was confirm by subculturing them alternately in EMB-free and EMB containing media. T organisms were grown in modified Youman's medium on rotary shaker at 37° Cells were harvested in their respective mid log phases which was 48 h for suso ptible strain and 72 h for resistant strain.

Isolation of cell wall and cell membrane fractions

Mycobacterial cells were disintegrated by ultrasonication, according to the meth of Hill and Ballou (1966). Subcellular fractions were isolated and purified by dif rential centrifugation according to the method of Kearney and Goldman (197 Purity of cell membrane was checked by measuring the activity of ATPase accordi to the method of Penumarti and Khuller (1983).

Labelling of phospholipids

Cells harvested in log phase were washed with normal saline and resuspended Kreb's Ringer buffer under sterile conditions. Cells were incubated at 37°C for under shaking conditions to get a homogenous suspension. (1-14C)-Sodium aceta (25 μCi/100 ml of medium) was added to cells. Incubation was continued for 90 m and at different time intervals 10 ml aliquots from each culture were transferred i tubes containing 0·5 ml of 1 M KCN (Kilburn et al., 1981). The tubes were cent fuged at 2,700 g for 15 min. The cell pellet was recovered and lipids extract Radioactivity was counted in a Packard Tricarb Liquid Scintillation Counter usin; toluene based scintillation fluid containing 0·4% (w/v) PPO and 0·05% (w POPOP.

Extraction and identification of lipids

Lipids were extracted and purified by the method of Folch et al. (1957). Individ phospholipid components were seperated by thin-layer chromatography (TLC) silica gel H plates using solvent system, chloroform:methanol:7N ammonia (65:25 v/v/v). Phospholipids were quantitated by estimating lipid phosphorus according the method of Bartlett (1959) as modified by Marinetti (1962). The acetone insolu phospholipids were used for fatty acid analysis (Khuller and Brennan, 1972). Met esters of phospholipid fatty acids were prepared by thionyl chloride procedure Prabhudesai (1978) and were resolved by Nucon Gas Chromatogram (Model 57

seperate tuberculostearic acid and oleic acid. Fatty acids were identified by comparasion of their retention times with authentic standards. The amounts of fatty acids were calculated by triangulation.

Results and discussion

As lipids constitute a major portion of the mycobacterial envelope, an attempt was made to study their role in development of drug resistance. A mutant of *M. smegmatis*, isolated by the replica plating method was found to be resistant to 200 μg/ml of EMB. Identical growth conditions were used for both parent and variant strains and they were harvested at similar phases of growth. Analysis of the total phospholipid (TPL) content of EMB-resistant mutant revealed (table 1) a significantly lower ($P \leqslant 0.001$) level when compared to the EMB-susceptible strain. This decrease was reflected in cardiolipin (CL) content, while there was no apparent change in the PE content. Another quantitative alteration observed in the EMB-resistant strain was its increased phosphatidylinositol mannoside (PIM) content ($P \leqslant 0.001$). Relative decrease in CL content of EMB-resistant strain is much more than augmentation in its PIM content which probably accounts for decreased TPL content of EMB-resistant strain. However, Cheema *et al.* (1986) observed no quantitative changes in phospholipid content of EMB-susceptible and EMB-resistant (resistant to 54 μg/ml EMB) strains. This inconsistency could be due to high level of resistance of mutant used in the present study.

Table 1. Comparison of phospholipid composition of EMB-susceptible and resistant strains of *M. smegmatis*.

Strain	Total phospholipids mg/g dry wt. of cells	Individual phospholipids (mg/g dry wt. of cells)		
		PIM	PE	CL
EMB-susceptible	25.89 ± 2.71	9.54 ± 1.74	3.21 ± 1.37	13.07 ± 1.94
EMB-resistant	19.76 ± 0.78^a	13.22 ± 1.39^a	2.59 ± 0.64	3.94 ± 0.37^a

Values are mean $\pm$ SD from 5 different batches.
$^a P \leqslant 0.001$.

Analysis of phospholipid fatty acids (table 2) revealed that the relative percentage of various fatty acids of the EMB susceptible strain differed from that of the EMB-resistant strain. A significant decrease in the proportion of myristic acid and a

Table 2. Relative percentage of phospholipid fatty acids in EMB-susceptible and resistant strains of *M. smegmatis*.

Strain	Fatty acid composition (relative percentage)						
	14:0	16:0	16:1	18:0	18:1	18:Me	U/S
EMB-susceptible	7.84	39.53	11.45	11.41	9.27	20.54	0.26
EMB-resistant	5.27	33.86	9.80	11.33	10.70	28.07	0.26

polar lipid fractions of different sensitive and resistant strains of mycobacteria. It was further suggested that differences could be found in the analysis of fatty acids in definitive subcellular fractions.

Since quantitative changes were observed in TPL content of EMB-susceptible and resistant strains, precursor incorporation studies were carried out to determine phospholipid synthesis in both the strains. Pulse labelling of lipids with $[1\text{-}^{14}C]$-sodium acetate followed for 90 min revealed that the incorporation into phospholipids increased continuously with time in susceptible as well as EMB-resistant strains (figure 1). However, the amount of radioactivity incorporated was significantly lower in EMB-resistant cells than susceptible cells. This explains the decreased TPL content of EMB-resistant strain as discussed earlier (table 1).
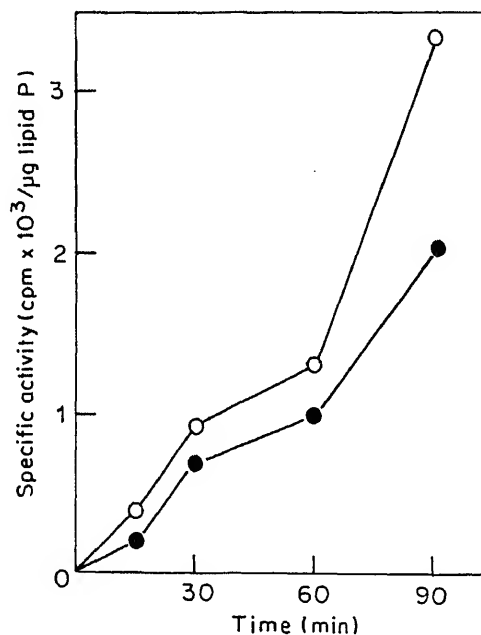
Figure 1. Change in specific activity of TPL of EMB-susceptible (○) and EMB-resistant (●) cells of *M. smegmatis* ATCC 607.

The cell membrane of the wild type strain of *M. smegmatis* contains more phospholipids than its cell wall and is enriched in PIM, as was also observed by Penumarti and Khuller (1983). On the contrary, cell wall of EMB-resistant strain contains more PL than its cell membrane. Unlike sensitive strain, PIM are equally distributed in the wall and membrane fraction of the EMB-resistant strain. Cell wall fraction of mutant strain showed an accumulation of phospholipids accompanied by decreased PL content in cell membrane, when compared with susceptible strain. Individual phospholipid content of cell wall of EMB-resistant strain was also enhanced (table 3).

und lular fraction	TPL (mg/g dry wt. of cells)	Individual phospholipids (mg/g dry wt. of cells)		
		PIM	PE	CL
...susceptible strain				
...wall	27.36 ± 2.97	8.99 ± 1.08	4.15 ± 0.73	14.21 ± 3.42
...membrane	50.67 ± 1.27	35.37 ± 1.57	3.15 ± 0.39	12.14 ± 1.20
...resistant strain				
...wall	65.65 ± 2.46^c	29.13 ± 2.55^c	10.37 ± 1.22^c	26.25 ± 0.57^b
...membrane	42.54 ± 4.75^a	31.56 ± 4.13	4.59 ± 0.51^b	6.38 ± 0.25^c

are mean ± SD from 3 independent batches.
5; $^bP \leqslant 0.01$; $^cP \leqslant 0.001$.

findings are analogous to those of Mackenzie and Jordon (1970) who observed cumulation of PL in cell envelope of viomycin resistant *Rhizobium melliloti*. pholipid fatty acid composition of EMB resistant cell wall fraction was also nt from that of EMB susceptible strain (table 4). There was a significant se in the level of unsaturated fatty acids in the cell wall of EMB-resistant st ain panied by increased level of saturated fatty acids. The decrease in unsaturated acids is revealed in the level of both palmitoleic acid and oleic acid. This nts for lower U/S ratio of EMB-resistant cell wall than that of the EMB-otible preparation. Increase in unsaturated fatty acids is known to increase the rane permeability, whereas increase in saturation decreases membrane perme- (McElhaney et al., 1973). Thus it can be assumed that a decrease in the un-tion of cell wall of mutant strain provides a barrier to penetration of drug ng in decreased sensitivity to the drug. Wada et al. (1975) also suggested that crease in the proportion of unsaturated fatty acids in phospholipids may be ated with increased polymyxin B sensitivity. Cell membrane of EMB-resistant exhibited an increase in the level of unsaturated fatty acids, particularly of acid resulting in increased U/S ratio in comparison to EMB-susceptible cell rane (table 4). Increased unsaturation of cell membrane of mutant strain ensated the decreased unsaturation of cell wall resulting in unaltered U/S ratio ole cell, as compared to EMB-susceptible strain. David (1980) proposed that nce in nontubercular mycobacteria is determined by the structure of bacterial

. Phospholipid fatty acid composition of subcellular fractions of EMB-susceptible and resistant *gmatis*.

	Subcellular fraction	Fatty acid composition (relative percentage)						
		14:0	16:0	16:1	18:0	18:1	18:Me	U/S
...susceptible	Cell wall	7·59	11·59	27·32	16·61	9·97	26·91	0·59
...resistant	Cell wall	9·98	26·84	10·64	35·94	Traces	9·98	0·13
...susceptible	Cell membrane	5·73	23·39	6·14	11·55	8·73	22·22	0·24

where transport actually takes place. The observations obtained in the presen[t] investigation also suggest that increase in phospholipid content of cell wall might b[e] causing a non specific blanketing action as hypothesised by Anderes *et al.* (1971) fo[r] antibiotic resistant *Psudomonas aeruginosa*. Decreased degree of unsaturation of ce[ll] wall phospholipid fatty acids also appear to be important in providing a barrier t[o] the penetration of drugs. As a result, the drugs are unable to penetrate into the ce[ll] which leads to decreased susceptibility towards drugs. However further studies o[n] uptake of labelled EMB by EMB-susceptible and resistant strain are necessary t[o] confirm this hypothesis.

Acknowledgement

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tion of myosin heavy chain genes during cardiac hypertrophy

N. SELVAMURUGAN, J. KAMALA and C. RAJAMANICKAM*

Department of Biochemistry, School of Biological Sciences, Madurai Kamaraj University, Madurai 625 021, India

Abstract. Nick translation of intact rat heart nuclei has shown that the incorporation of [³H]–dATP is greater in hypertrophic heart nuclei than in normal heart nuclei suggesting that hypertrophic heart nuclei have more DNase I sensitive regions than normal heart nuclei. DNase I sensitivity analysis has shown that the rate and extent of digestion of myosin heavy chain genes are greater in hypertrophic than in normal heart nuclei. Dot blot hybridization analysis of myosin heavy chain transcripts from hypertrophic heart nuclei using myosin heavy chain cDNA as probe has shown that the sensitivity of myosin heavy chain genes to DNase I in hypertrophic heart nuclei correlates with myosin heavy chain gene activation and increased number of transcripts.

Keywords. Cardiac hypertrophy; nick translation of intact nuclei; DNase I digestion pattern; myosin heavy chain genes.

ction

: hypertrophy is a basic adaptive response of the heart to any increased
al demand. The development of cardiac hypertrophy is associated with
d transcription and translation, and a consequent increase in the ribosomal
oly(A)-containing RNA, and myofibrillar and cytoplasmic protein contents
ac muscle cells (Zak and Rabinowitz, 1979). The increase in RNA synthesis
an altered conformation of chromatin (Limas, 1982). The regions of
tin that are engaged in transcription and those that are not, generally show a
tial sensitivity to DNase I digestion (Weintraub and Groudine, 1976).
gh it is clear that the transcriptionally active regions of the chromatin are
ned in a conformation distinguishable from that of the transcriptionally inert
tin, the nature of the changes which accompany gene activation remains
.

biting the sensitivity of transcriptionally active chromatin to DNase I, we
out the nick-translation of intact nuclei obtained from both normal and
ophic hearts at low concentrations of DNase I, so that the active genes are
rather than cleaved (Levitt *et al.*, 1979). In DNase I sensitivity experiments,
rom normal and hypertrophic heart were compared in terms of the rate and
of digestion of the chromatin. At the more specific gene level we have
ed to study the activation of myosin heavy chain genes which is responsible
or changes in the Ca-dependent ATPase activity of myosin (Lompre *et al.*,
uring cardiac hypertrophy.

Cardiac hypertrophy in female albino Wistar rats was induced by following the method of Rakusan and Poupa (1966) with minor modifications. A tantalum hemoclip (Edward Weck and Co., Cat. No. 523135) was placed around the proximal ascending aorta just distal to the coronary ostis. A sham operation was performed on control animals and no band was placed around the ascending aorta. The development of hypertrophy was calculated as per cent increase in the ratio of heart weight (wet wt.) to body weight with respect to sham-operated controls (Meenakshi *et al.*, 1983).

Isolation and nick translation of nuclei

The isolation of nuclei was carried out by the method of Jackowski and Liew (1980). The nuclear pellet was purified by pelleting through a step gradient of 2·4 and 1·6 M sucrose at 100,000 g for 1 h at 4°C in a Hitachi SCP 85H centrifuge. The DNA content of the nuclei was determined by Burton's (1956) procedure. Nuclei were pelleted and rinsed in nick translation buffer (50 mM Tris pH 7·9, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol and 10 μg/ml BSA) and then used for nick translation as described by Levitt *et al.* (1979).

DNase I sensitivity studies

Nuclei from sham-operated and hypertrophic hearts were suspended in 50% w/v glycerol, 10 mM Tris pH 7·4, 10 mM NaCl and 3 mM $MgCl_2$, and aliquots digested with various concentrations of pancreatic DNase I (Sigma) at 37°C for 5 min (Dimitriadis and Tata, 1980). DNA extracted after DNase I treatment was run on a 1% agarose gel. The pattern of digestion was analysed by electrophoresis on a 12% denaturing polyacrylamide gel (Noll, 1974). The gel was stained in 0·005% Stains-all in 50% formamide, destained in water, and scanned in an LKB Ultrascan.

Dot hybridization analysis

Plasmid pcMHC 5, a pBR322 derivative containing myosin heavy chain cDNA from rat heart (Mahdavi *et al.*, 1982), and plasmid pPC-P450–91, a pUC9 derivative containing cytochrome P450e cDNA from rat liver (Ravisankar and Padmanaban, 1985) were nick-translated as described by Rigby *et al.* (1977). Total nuclear RNA was isolated from sham-operated and hypertrophic hearts as described by Penmann (1966). Spotting of nucleic acids on nylon filters and hybridization with nick-translated probes (specific activity 1×10^8 cpm/μg DNA) were done according to the protocols given by the manufacturer. Quantitative analysis of dot blots was done by cutting out the radioactive spots on the nylon filter and counting in a liquid scintillation counter.

Extending this initial observation, we have studied gene activation in hypertrophic heart in general as well as at a specific gene level (myosin heavy chain) during cardiac hypertrophy. The advantage of nick translation studies of intact nuclei is that an active transcriptional state of chromatin is indicated by increased incorporation of label in the nick translation reaction because of the higher DNase I sensitivity of "active" chromatin. This was shown by hybridizing the nick-translated DNA with total cellular RNA (Levitt et al., 1979). Nick translation studies have also been useful in localizing DNase I sensitive regions of chromatin in interphase nuclei and in dividing cells (Hutchison and Weintraub, 1985). The increased incorporation of [^{3}H]–dATP in hypertrophic heart nuclei reflects the activation of regions of chromatin for transcription (table 1). This could be due to changes in the conformation of transcribed regions of chromatin, which then become more accessible to regulatory proteins, nucleases and polymerases.

Table 1. Nick translation of nuclei.

Source of nuclei	Incorporation of [^{3}H]–dATP (cpm/μg DNA)
Sham-operated heart	$7.7 \pm 0.3 \times 10^4$
Hypertrophic heart	$10.0 \pm 0.4 \times 10^4$

All values are mean $\pm$ SD of mean of 3 experiments.

DNase I digestion pattern

Increasing the DNase I concentration in a DNase I digestion reaction results in active nuclear DNA sequences being rapidly digested and solubilized. When nuclei from sham-operated and hypertrophic hearts were treated with various concentrations of DNase I (0, 2, 10 and 50 U/mg DNA) for a constant incubation time (5 min) and the extracted DNA electrophoresed on neutral 1% agarose gel and stained with ethidium bromide, different patterns were observed (figure 1). At various DNase I concentrations, DNA of hypertrophic heart nuclei was found to be digested to a greater extent than DNA of control sham-operated heart nuclei. Further, the rate of production of smaller fragments was faster in hypertrophic heart nuclei (data not shown). When the DNA fragments obtained after DNase I treatment (150 U/mg DNA for 5 min) of sham-operated and hypertrophic heart nuclei were analysed by denaturing PAGE, the usual ladder-like pattern of bands differing in chain length by 10 nucleotides was observed (figures 2 and 3). An intense band at 80 nucleotides reflects the periodicity of the DNA superhelix in the nucleosome. The 10-bp-interval cleavage pattern with DNase I was obtained for both sham-operated and hyper-trophic heart nuclei but the production of smaller fragments was more in hypertrophic heart nuclei. This reflects a greater accessibility of the DNA in the
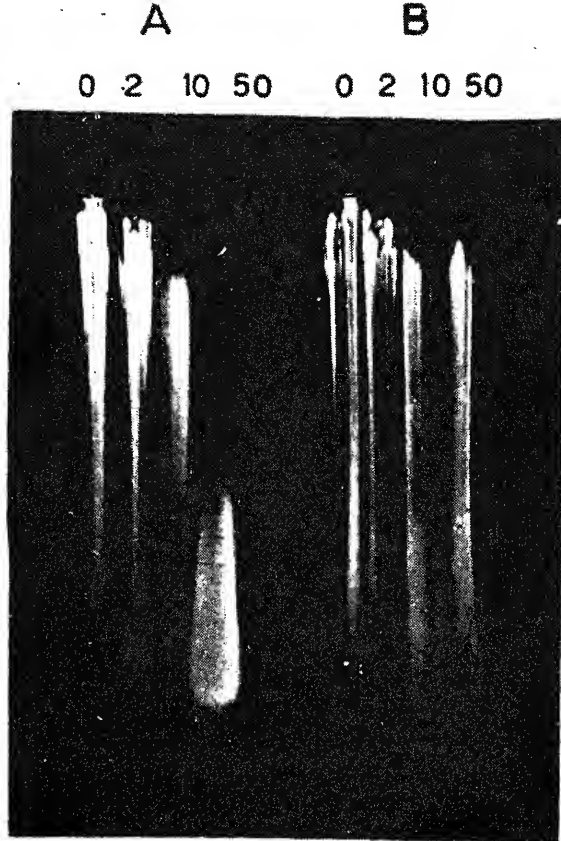
Figure 1. Agarose gel electrophoresis of the DNA released after DNase I digestion. Nuclei from (A) hypertrophic heart and (B) sham-operated heart were incubated with increasing concentrations (U/mg DNA) of DNase I and DNA was extracted after 5 min.

chromatin of hypertrophic heart nuclei to DNase I. Fragments smaller than [nucleotides] are not seen in the photograph (figure 2) probably because of the low efficiency of precipitation of the fragments by ethanol (Sollner-Webb and Felsenfeld, 1977).

The specific cutting of DNA in chromatin is an intrinsic property of the DNA double helix and the frequency of cutting is dependent on the exposure of the potential sites to nuclease attack (Lutter, 1978). It has been shown that the rate and extent of digestion by DNase I are greater in chromatin obtained from young (18–20 weeks) rat brain than in that obtained from adult (90–97 weeks) brain (Chaturvedi and Kanungo, 1985). DNase I sensitive sites have been found to be stabilized by high mobility group proteins 14 and 17 (Nicolas *et al.*, 1983). In an earlier report we have shown that 0·35 M NaCl extractable proteins contribute to the enhanced DNase I sensitivity of hypertrophic heart nuclei (Kamala *et al.*, 1986).
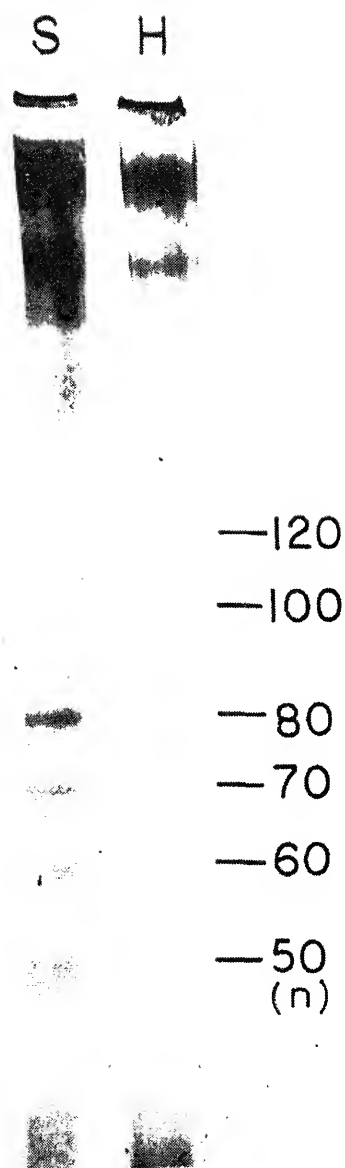
Figure 2. DNA fragments obtained after DNase I treatment of nuclei of sham-operated (S) and hypertrophic (H) hearts. Numbers are fragment sizes (n, nucleotides).

robe (pcMHC 5) clearly indicate that the extent of hybridization of the probe with DNA from DNase I treated hypertrophic heart nuclei was very much reduced ompared to that with total undigested DNA from hypertrophic heart nuclei (figure , C and D). In contrast, hybridization with DNA from sham-operated heart nuclei
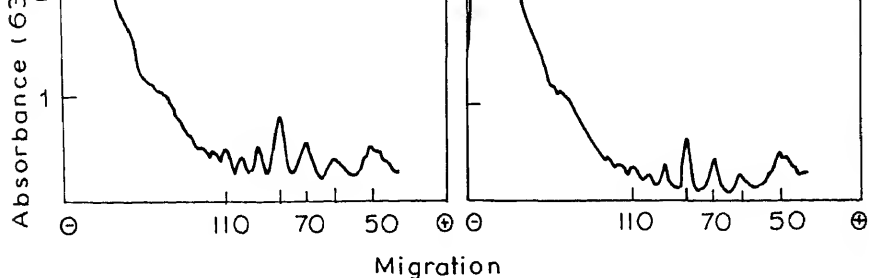
Figure 3. Densitometric scans of DNA fragments produced by DNase I.
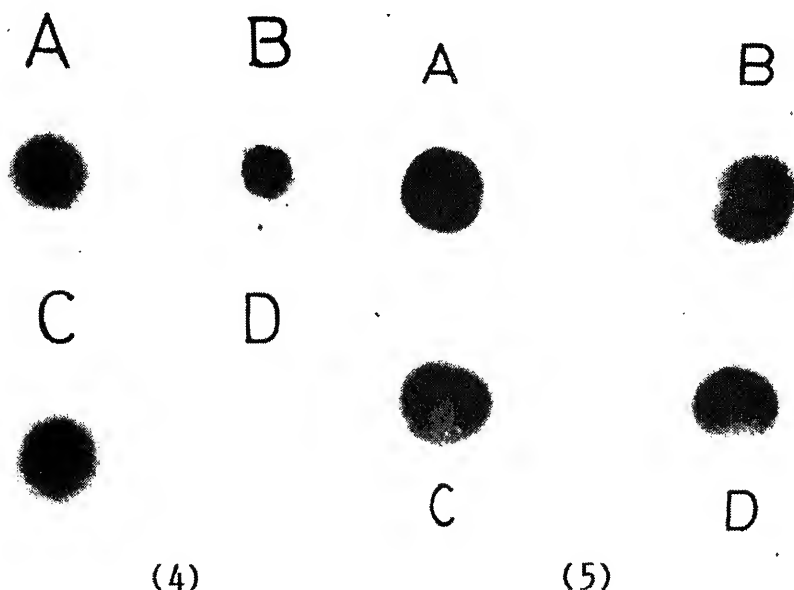
Figures 4 and 5. Dot blot hybridization of DNA from untreated and DNase I treated sham-operated and hypertrophic heart nuclei with nick-translated (4) myosin heavy chain cDNA probe (pcMHC 5) and (5) cytochrome P450e cDNA probe (pPC-P450-91). (A), Sham-operated heart nuclei; (B), sham-operated heart nuclei + DNase I (10% acid solubility); (C), hypertrophic heart nuclei; (D), hypertrophic heart nuclei + DNase I (10% acid solubility).

phenobarbitone in rat liver but the gene is not active in heart. The results therefore indicate the specific activation of myosin heavy chain genes in hypertrophic heart nuclei.

Increased nuclease sensitivity appears to be an important criterion for confirming the transcriptionally open conformation of chromatin. We also checked whether the increased DNase I sensitivity of myosin heavy chain genes in hypertrophic heart

Figure 6. Dot blot hybridization of nuclear RNA from sham-operated and hypertrophic heart nuclei with nick-translated myosin heavy chain cDNA probe (pcMHC 5). (A), RNA from sham-operated heart nuclei; (B), RNA from hypertrophic heart nuclei.

i can be correlated with increase in myosin heavy chain transcripts in the nuclei. re 6 shows the dot blot hybridization of nuclear RNA extracted from sham-opera- nd hypertrophic heart nuclei with the myosin heavy chain cDNA probe. The idization signal observed in the RNA sample from hypertrophic heart nuclei stronger than that in the RNA from sham-operated heart nuclei. This result and esults shown in figure 4 establish a correlation between DNase I sensitivity of sin heavy chain genes in hypertrophic heart nuclei and their transcriptionally e state. There are, however, instances where DNase I sensitivity is not correlated active transcription of genes (Stalder *et al.*, 1980). In the case of vitellogenin s also, DNase I sensitivity parallels transcriptional activation (Folger *et al.*,). DNase I sensitivity is not restricted to the coding regions of the genes but ids upstream and downstream (Strobe *et al.*, 1981). Koropatnick and Duerksen 7) showed that protein-encoding DNA sequences that are available for cription are more sensitive to DNase I than those which are unavailable for cription and that the increase in nuclease sensitivity is detected in actively cribed metallothionenin-I and α-fetoprotein genes in embryonic liver. Our ts show that the activation of myosin heavy chain genes detected by DNase I tivity can be correlated with increase in myosin heavy chain transcripts. It ars that the change in chromatin structure of specific genes during cardiac rtrophy is associated with the transcriptional state.

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Effect of carnitine administration on levels of lipid peroxides and activities of superoxide dismutase and catalase in isoproterenol-induced myocardial infarction in rats

S. SUSHAMA KUMARI and VENUGOPAL P. MENON*

Department of Biochemistry, University of Kerala, Kariavattom, Trivandrum 695 581, India

Abstract. The effect of carnitine administration on levels of lipid peroxide and activities of superoxide dismutase and catalase was studied in rats administered isoproterenol to induce myocardial infarction. Levels of fatty acid were lower in rats pretreated with carnitine at the peak period and given isoproterenol than the levels in isoproterenol-treated control rats. Lipid peroxides were decreased in the heart at peak infarction in carnitine-treated rats compared to the levels in isoproterenol-treated controls. Activities of superoxide dismutase and catalase showed no change in carnitine-treated animals given isoproterenol compared to those in normal control rats, while they decreased in animals treated with isoproterenol alone.

Keywords. Malondialdehyde; hydroperoxides; conjugated dienes; superoxide dismutase; catalase; free fatty acids.

Introduction

A number of substances have been identified for their ability to protect against experimental myocardial infarction induced by a β-agonist isoproterenol (Wexler and Greenberg, 1978). Any substance which can prevent an attack or accelerate the process of recovery will have considerable clinical application.

Carnitine (L-3-hydroxy-4-trimethylammonium butyrate) which plays an important role in the transmembrane transport of long chain fatty acids for their oxidation in the mitochondria, has been reported from our laboratory to offer protection against myocardial infarction induced by isoproterenol (Saleena *et al.*, 1986). The molecular events underlying this phenomenon are not clear. There are some reports indicating that the level of carnitine decreases in the myocardium during ischemia (Whitmer *et al.*, 1978).

Recently we reported that one of the major events taking place during myocardial infarction is the increase in levels of myocardial lipid peroxides which may cause damage to the myocardium (Sushama and Menon, 1987). The primary substrates for the formation of lipid peroxides are fatty acids and long chain acyl coenzyme A derivatives whose concentrations have been reported to increase during myocardial infarction (Gudjarnason, 1980).

In view of the observed protective action of carnitine it was thought worthwhile to study its effect on levels of lipid peroxides in animals pretreated with carnitine and administered isoproterenol. Changes in activities of two enzymes closely associated with the process, namely superoxide dismutase (SOD) and catalase, have also been studied. The results of these investigations are reported in this communication.

(2) carnitine-treated group.

DL-Carnitine in physiological saline was administered daily intramuscularly at a dose of 10 mg/100 g body weight for 10 days. The control group received physiological saline. At the end of the period, the animals were regrouped as follows.

1. Normal control group.
2. Normal administered isoproterenol.
3. Carnitine-treated control group.
4. Carnitine-treated rats administered isoproterenol.

Isoproterenol was given at a dose of 35 mg/100 g body weight in two injections 24 h apart as previously described (Saleena *et al.*, 1982). Rats in groups 3 and 4 continued to receive carnitine. Rats of group 4 were given isoproterenol 3 h after administration of carnitine.

The isoproterenol-treated rats showed signs of shock tachycardia, dyspnea, rapid respiration, etc. The animals of group 4 showed these signs to a lesser extent. The surviving animals of group 4 continued to receive carnitine till the end of the experiment. Rats in each group (45 rats) were killed after overnight starvation at 5 h, 36 h and 7·5 days after the first injection. The control rats (15) were sacrificed along with those killed at 5 h. The heart tissue was removed to ice-cold containers for various estimations. Serum creatine phosphokinase (CPK), glutamate oxalotransaminase (GOT) and glutamine pyruvic transaminase (GPT), free fatty acid levels in the heart and serum, activities of SOD and catalase in the heart, and levels of malondialdehyde, hydroperoxides and conjugated dienes in the heart were estimated as described earlier (Sushama and Menon, 1987). Statistical analysis was carried out using Student's '*t*' test (Bennet and Franklin, 1967).

Results

The rate of survival in rats given isoproterenol alone was 60–65% while in the case of rats pretreated with carnitine and then given isoproterenol it was 85–90%. These results are from 5 experiments using 25 rats in each group.

Serum GOT, GPT and CPK

Rats treated with isoproterenol showed significantly higher values for GOT and GPT at 5 h, 36 h and 7·5 days after injection than normal rats. In the case of CPK, the values were higher only at the peak period (table 1).

Rats treated with carnitine before isoproterenol injection showed lower enzyme activities at peak period of infarction than the isoproterenol-treated animals. Carnitine alone did not bring about any significant change in the enzyme activities.

Levels of free fatty acid in serum and heart

The data are given in table 2. The levels of free fatty acid registered an increase at

	Time after first injection	GOT μmol oxalo-acetate formed/min/l of serum	GPT μmol pyruvate formed/min/l of serum	CPK μmol creatine formed/min/l of serum
mal control	—	150·68 ± 4·6	60·45 ± 3·75	270·4 ± 12·66
roterenol	5 h	201·6 ± 12·24[+]	110·22 ± 2·86*	292·6 ± 10·92
	36 h	365·6 ± 10·24	202·3 ± 8·48*	788·34 ± 24·86*
	7·5 days	190·45 ± 8·25	70·46 ± 3·29	198·44 ± 10·39[+]
nitine control	—	149·46 ± 8·60	62·46 ± 4·16	260·46 ± 17·24
nitine + isoproterenol	5 h	190·34 ± 6·10*	85·45 ± 4·44*	285·44 ± 10·39
	36 h	301·4 ± 8·28‡*	152·3 ± 3·98‡*	510·4 ± 20·68‡*
	7·5 days	180·65 ± 12·33	65·34 ± 3·82	201·3 ± 10·01[+]

; 2 to 4 compared with group 1.

4 has been compared with group 2.

01; [+]P between 0·01 and 0·05; ‡$P < 0.01$.

given are the mean from 7 rats in each group ± SEM.

Table 2. Changes in the free fatty acids in serum and heart at different stages of infarction.

Group	Time after first injection	Free fatty acids mg/100 ml serum	Free fatty acids mg/100 g heart tissue
1. Normal control	—	86·2 ± 5·64	603·6 ± 12·48
2. Isoproterenol	5 h	198·24 ± 10·23*	592·6 ± 25·68
	36 h	165·66 ± 8·44*	1389·2 ± 40·24*
	7·5 days	129·28 ± 7·48*	546·24 ± 16·48
3. Carnitine control	—	90·48 ± 2·76	690·8 ± 20·66‡
4. Carnitine + isoproterenol	5 h	160·22 ± 5·82*	780·5 ± 30·48*
	36 h	128·24 ± 4·66*‡	1015·9 ± 20·46
	7·5 days	112·62 ± 4·28*	596·8 ± 20·46

Groups 2 to 4 have been compared with group 1.

Group 4 has been compared with group 2.

*$P < 0.01$; ‡$P < 0.01$.

Values given are the mean from 7 rats in each group ± SEM.

period of infarction in both serum and heart in rats administered isoproterenol. case of serum the fatty acid levels showed a sudden spurt at 5 h. In the case of retreated with carnitine and given isoproterenol, the levels of free fatty acid at infarction in both serum and heart were lower than those in animals given oterenol alone. In the carnitine group the level of free fatty acid at 5 h in the was higher than the level in animals treated with isoproterenol. The carnitine-d animals also showed higher level of free fatty acids in the heart than normal ol rats.

the 5 and 36 h in animals administered isoproterenol alone. On the other hand...
pretreated with carnitine and then administered isoproterenol showed signifi...
decrease in the level at the 5 and 36 h intervals (table 3).

Table 3. Changes in the levels of lipid hydroperoxides, conjugated dienes and malondialdehyde in...
at different stages of infarction.

Group	Time after first injection	Hydroperoxides	Conjugated dienes	Malondialde...
		. . . . mM/100 gm tissue.		
1. Normal control	—	$2 \cdot 80 \pm 0 \cdot 18$	$4 \cdot 37 \pm 0 \cdot 26$	$1 \cdot 20 \pm 0 \cdot 0...$
2. Isoproterenol	5 h	$16 \cdot 77 \pm 0 \cdot 57*$	$12 \cdot 5 \pm 0 \cdot 60*$	$2 \cdot 13 \pm 0 \cdot 1...$
	36 h	$20 \cdot 11 \pm 1 \cdot 12*$	$5 \cdot 86 \pm 0 \cdot 30*$	$2 \cdot 89 \pm 0 \cdot 1...$
	7·5 days	$2 \cdot 27 \pm 0 \cdot 11$	$2 \cdot 83 \pm 0 \cdot 14$	$1 \cdot 12 \pm 0 \cdot 0...$
3. Carnitine control	—	$2 \cdot 98 \pm 0 \cdot 15$	$4 \cdot 28 \pm 0 \cdot 13$	$1 \cdot 41 \pm 0 \cdot 0...$
4. Carnitine + isoproterenol	5 h	$3 \cdot 03 \pm 0 \cdot 19\ddagger$	$5 \cdot 75 \pm 0 \cdot 17*\ddagger$	$1 \cdot 52 \pm 0 \cdot 0...$
	36 h	$3 \cdot 52 \pm 0 \cdot 14\ddagger$	$4 \cdot 92 \pm 0 \cdot 25\ddagger$	$1 \cdot 16 \pm 0 \cdot 0...$
	7·5 days	$3 \cdot 12 \pm 0 \cdot 10$	$3 \cdot 84 \pm 0 \cdot 17\ddagger$	$1 \cdot 09 \pm 0 \cdot 0...$

Groups 2 to 4 have been compared with group 1.
Group 4 has been compared with group 2.
$*P < 0 \cdot 01$; $\ddagger P < 0 \cdot 01$.
Values given are the mean from 7 rats $\pm$ SEM.

Activities of SOD (EC 1·15·1·1) and catalase (EC 1·11·1·6)

The activities of SOD and catalase showed slight increase at 5 h and then fell b...
the normal levels at peak infarction in animals administered isoproterenol (tabl...
In animals administered isoproterenol after pre-treatment with carnitine, activit...
SOD showed slight elevation at 5 h and then fell to values near normal at p...

Table 4. Activities of SOD and catalase in heart at different stages of infarction.

Group	Time after first injection	SOD (units*/mg protein)	Catalase ($\times 10^{-3}$ units*/ protein)
1. Normal control	—	$11 \cdot 08 \pm 0 \cdot 85$	$7 \cdot 18 \pm 0 \cdot 2...$
2. Isoproterenol	5 h	$15 \cdot 88 \pm 0 \cdot 64*$	$8 \cdot 45 \pm 0 \cdot 4...$
	36 h	$6 \cdot 05 \pm 0 \cdot 35*$	$5 \cdot 68 \pm 0 \cdot 2...$
	7·5 days	$9 \cdot 16 \pm 0 \cdot 38$	$6 \cdot 82 \pm 0 \cdot 3...$
3. Carnitine control	—	$11 \cdot 08 \pm 0 \cdot 48$	$8 \cdot 25 \pm 0 \cdot 5...$
4. Carnitine + isoproterenol	5 h	$16 \cdot 19 \pm 0 \cdot 84*$	$10 \cdot 03 \pm 0 \cdot 4...$
	36 h	$12 \cdot 12 \pm 0 \cdot 48\ddagger$	$8 \cdot 76 \pm 0 \cdot 2...$
	7·5 days	$13 \cdot 16 \pm 0 \cdot 78\ddagger$	$8 \cdot 83 \pm 0 \cdot 2...$

Groups 2 to 4 have been compared with group 1.
Group 4 has been compared with group 2.
$*P < 0 \cdot 01$; $+P$ between $0 \cdot 01$ and $0 \cdot 05$; $\ddagger P < 0 \cdot 01$.
*Unit = enzyme concentration required to inhibit the optical density at 560 n...

t elevation above the activity in normal rats.

~ussion

~se results confirm our earlier observation that carnitine offers some protection to ~ myocardium during isoproterenol-induced myocardial infarction. An earlier ~y showed that although there was some increase in the activities of serum CPK ~ serum GOT, histopathological results indicated that the degree of necrosis was ~imal in rats pretreated with carnitine before the administration of isoproterenol ~eena et al., 1986).

~he present study indicates a decrease in the levels of hydroperoxides, conjugated ~es and malondialdehyde in animals pretreated with carnitine before the admini-~tion of isoproterenol. Lipid peroxidation begins with the formation of a lipid free ~cal, which rearranges to form a diene. Partial oxidation results in the formation ~ lipid peroxy radical which takes up a hydrogenation to form lipid hydroperoxide ~ipid endoperoxide. Malondialdehyde is a breakdown product of unsaturated ~ acids. The low levels of lipid peroxides in animals pretreated with carnitine ~gest that the protective action of carnitine may be due to its effect in decreasing ~ levels of lipid peroxides in the heart. In this context it has been reported that ~ng ischemia there is depletion of myocardial carnitine (Whitmer et al., 1978). But ~ not clear as to how the administered carnitine can cause reduction in levels of ~ peroxides. The substrates for microsomal peroxidation are fatty acids. The ~ortant function of carnitine is in the transmembrane transport of fatty acids ~oss the mitochondrial membrane for oxidation. It has also been reported that ~ acids and cholesterol esters increase during ischemia resulting in the disruption ~ell membrane (Whitmer et al., 1978). Administration of carnitine may help in the ~sport of fatty acids into the mitochondria resulting in the decrease in their level ~eak infarction. The increased level of free fatty acids in serum in animals treated ~ isoproterenol is due to increased lipolysis (Saleena et al., 1981). The decreased ~ls of free fatty acids in serum at peak infarction in animals pretreated with ~nitine may be due to decreased lipolysis, increased uptake by mitochondria, or ~h.

~ssociated with these changes we have also observed that the activities of two key ~ymes, SOD and catalase, are decreased in rats given isoproterenol, while in ~mals pretreated with carnitine before isoproterenol administration, the activities ~hese enzymes are comparable with those in normal control rats. Both these ~ymes help in scavenging toxic intermediates of incomplete oxidation in the body. ~ecrease in the activities of these enzymes can result in the formation of O_2^- and ~O_2, which in turn can form the hydroxyl radical ($OH^.$) which can participate in a ~nber of toxic reactions. The reason for the decrease in the activities of SOD and ~alase in the carnitine-treated animals is not clear. This may be due to decreased ~ocardial cell damage in the carnitine-treated animals. In this context, it has been ~erved by Burton (1985) that isolated perfused rabbit interventricular septa ~ treated with SOD can withstand ischemia (1 h) with little structural damage.

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Similar effects of β-alanine and taurine in cholesterol metabolism

A. N. LAKSHMI PRABHA, S. LEELAMMA and P. A. KURUP*

Department of Biochemistry, University of Kerala, Kariavattom, Trivandrum 695 581, India.

Abstract. β-Alanine, though producing a deficiency of taurine in the tissues, had a similar effect on cholesterol metabolism as taurine. Both caused increased activity of hepatic hydroxymethylglutaryl coenzyme A reductase and increased incorporation of 1, 2 of $[^{14}C]$-acetate into liver cholesterol. Both caused increased concentration of biliary cholesterol and bile acids. There was increased activity of lipoprotein lipase in heart, but decreased activity in the adipose tissue in both cases. Release of lipoproteins into circulation was decreased in both cases.

Keywords. β-Alanine; taurine; cholesterogenesis; biliary bile acids; lipoprotein lipase.

Introduction

Taurine is reported to be present in high concentrations in all mammalian tissues. Especially high concentrations are present in the cardiac tissue. Despite this widespread distribution and the high concentration in which it occurs, almost nothing is known about the function of this amino acid in metabolism, except for a report that taurine enhanced low density lipoprotein (LDL) receptor activity in cultured Hep. G2 cells (Stephen *et al.*, 1987). The difficulty in producing experimental taurine deficiency has been the major handicap in the study of the metabolic role of this simple substance. The observation that taurine levels were decreased in the tissues in rats administered taurine transport antagonists such as guanidinoethyl-sulphonate, β-alanine and hypotaurine (Shaffer and Kocsis, 1978) was expected to be of value in studying its metabolic role.

It was reported that cardiac muscle is depleted of its taurine stores in isoproterenol-induced myocardial infarction (Huxtable *et al.*, 1980). We have already shown that the concentration of cholesterol in the heart is significantly elevated in isoproterenol-induced myocardial infarction in rats (Saleena *et al.*, 1981). These observations led us to study the changes in the metabolism of cholesterol in the heart in taurine deficiency induced by β-alanine in rats. Quite unexpectedly it was observed in these studies that β-alanine, though causing a reduction in the level of taurine in many tissues including heart, functioned as a taurine agonist and functionally replaced the depleted taurine as far as the metabolism of cholesterol was concerned. The results which led to this conclusion are presented in this paper.

Materials and methods

Male albino rats (Sprague-Dawley strain, average body weight 150 g) were divided

Group 3 — taurine group.

The rats were fed a diet which had the following composition (g/100 g diet).

Corn starch   —   71
Casein (vitamin-and starch-free)   —   16
Groundnut oil   —   8
Vitamin mixture   —   1
Salt mixture   —   4

The vitamin mixture and the salt mixture used had the same composition as described earlier (Thomas et al., 1983). β-Alanine and taurine dissolved in water were administered orally by tube daily to the rats of groups 2 and 3, respectively, the former at a dose of 350 mg/100 g body weight (Shaffer and Kocsis, 1978) and the latter at 100 mg/100 g body weight (Sturman, 1973). The duration of the experiment was 7 days. At the end of this period, the rats were deprived of food overnight, stunned by a blow at the back of the neck, and killed by decapitation. Serum and tissues were removed to ice-cold containers for the estimation of taurine, lipids, bile acids and activities of the enzymes. A separate experiment with 12 rats in each group under similar conditions was carried out to study the release of lipoproteins into the circulation and the incorporation of labelled acetate into hepatic cholesterol.

Estimation of taurine was carried out by the procedure of Parker (1980). Cholesterol, triglycerides and phospholipids were estimated in the heart and liver as described before (Menon and Kurup, 1976). Hydroxymethylglutaryl coenzyme A (HMG CoA) reductase (EC $1\cdot1\cdot1\cdot34$) of liver was estimated as described by Venugopala Rao and Ramakrishnan (1975) by determining the ratio of HMG CoA to mevalonic acid. Incorporation of 1, 2-[^{14}C]-acetate into cholesterol in vivo in the liver was carried out as described before (Thomas et al., 1983). Ten μCi of labelled acetate per 100 g body weight was administered to the rats. Release of lipoproteins into the circulation was studied at the end of the experimental period using Triton WR 1339 to block the uptake of lipoproteins from the circulation by extrahepatic tissues (Schurr et al., 1972). Triton WR 1339, 50 mg/100 g body weight, was injected intraperitoneally in normal saline to overnight-fasted rats, and 4 hours later, blood was collected. Control animals received the same volume of normal saline instead of Triton. Serum was separated and cholesterol estimated as described above. Extraction of liver for bile acids was carried out according to the procedure of Okishio et al. (1967) and bile acids were estimated enzymatically using 3α-hydroxysteroid dehydrogenase (Robert, 1969). Estimation of bile acids and cholesterol in the bile was also carried out. The rats were anesthetized with pentathol sodium (5 mg/100 g body weight) and the bile duct was cannulated. The bile was continuously collected in an ice-cold tube for 2 h. Lipoprotein lipase (EC $3\cdot1\cdot1\cdot3$) activity of heart and adipose tissue was estimated according to the procedure of Krauss et al. (1974). Protein was estimated after TCA precipitation by the method of Lowry et al. (1951).

Statistical analysis was carried out by using Student's 't' test.

given β-alanine had lower levels of taurine in these tissues, the decrease in the
being much more than that in the heart (table 1). These results are in agreement
those reported by Shaffer and Kocsis (1978), who also found greater decrease in
ver than in the heart.

Table 1. Effect of administration of β-alanine and
taurine on level of taurine in heart and liver of rat.

Tissue	Taurine (mg/100 g wet tissue)		
	Group 1	Group 2	Group 3
Heart	388.6 ± 9.7	360.4 ± 7.6^b	402.1 ± 11.3
Liver	60.7 ± 1.5	29.0 ± 0.6^a	125.0 ± 3.6^a

Values are mean $\pm$ SE, of mean of results from 6 rats.
Group 1, control rats; group 2, β-alanine treated rats;
group 3, taurine treated rats.
Significance of difference for groups 2 and 3 vs group 1.
$^a P < 0.01$; $^b 0.01 < P < 0.05$.

lministration of taurine elevated the level of taurine in the liver and heart, the
ase being more in the liver than in the heart. A similar increase in taurine levels
sues was reported by Lombardini and Medina (1978).

entration of cholesterol, triglycerides and phospholipids in the heart and liver

results are shown in table 2. Concentration of cholesterol in the heart in both β-
ne and taurine groups was lower than that in control rats, while that in the liver
not significantly altered. Total phospholipids also decreased in the heart in both
nine and taurine groups but increased in the liver. Triglycerides on the other
increased in the heart in both the groups, but decreased in the liver.

Table 2. Effect of administration of β-alanine and taurine on levels of cholesterol,
triglycerides and phospholipids in heart and liver of rat.

	Tissue	Cholesterol/triglycerides/phospholipids (mg/100 g wet tissue)		
		Group 1	Group 2	Group 3
Cholesterol	Heart	168.4 ± 4.6	137.6 ± 3.4^a	93.6 ± 2.2^a
	Liver	293.2 ± 7.0	309.7 ± 8.4	280.2 ± 5.7
Triglycerides	Heart	45.0 ± 1.0	56.3 ± 1.4^a	76.3 ± 2.1^a
	Liver	485.2 ± 14.1	335.3 ± 7.7^a	362.9 ± 9.4^a
Phospholipids	Heart	2685 ± 183	2073 ± 135^a	1104 ± 70^a
	Liver	2923 ± 178	3876 ± 252^a	3566 ± 225^b

than in control group (table 3).

Table 3. Effect of administration of β-alanine and taurine on activity of hepatic HMG CoA reductase and incorporation of [^{14}C]-acetate into cholesterol in rat.

Group	Activity of hepatic HMG CoA reductase (ratio of HMG CoA to mevalonate)*	Incorporation of [^{14}C]-acetate into cholesterol (cpm/g tissue)
1	3.00 ± 0.08	1022 ± 63
2	2.15 ± 0.05^a	1898 ± 123^a
3	1.67 ± 0.038^a	3672 ± 250^a

*Decreased ratio indicates increased enzyme activity. Other details as in table 1.

Concentration of hepatic and biliary bile acids and biliary cholesterol

Both β-alanine and taurine treated rats showed significant increase in the concentration of biliary bile acids and cholesterol (table 4). But concentration of hepatic bile acids was lower in both the groups than in the control group.

Table 4. Effect of administration of β-alanine and taurine on concentration of hepatic and biliary bile acids and bile cholesterol in rat.

Group	Hepatic bile acids (mg/100 g tissue)	Biliary bile acids (mg/100 ml bile)	Biliary cholesterol (mg/100 ml bile)
1	34.25 ± 0.96	73.70 ± 1.62	4.30 ± 0.09
2	26.19 ± 0.63^a	119.1 ± 3.22^a	18.20 ± 0.46^a
3	18.73 ± 0.43^a	105.6 ± 2.75^a	14.60 ± 0.35^a

Details as in table 1.

Thus both β-alanine and taurine cause increased cholesterol synthesis in the liver as is evident from the increased incorporation of label into liver cholesterol and the higher activity of HMG CoA reductase. Increased concentration of biliary bile acids in both these groups indicates increased bile acid synthesis from cholesterol in the liver. Taurine, by forming tauroconjugates, may remove more bile acids from the liver into the bile. The removal of bile acids from the liver may result in increased degradation of more cholesterol.

In this connection the report of Stephen *et al.* (1987) that taurine enhanced LDL receptor activity in cultured Hep. G2 cells is pertinent. Stimulation of LDL receptor activity was also obtained with 10 mM cysteine, a taurine precursor. Increased cellular concentration of taurine and cysteine was associated with an increased rate of bile acid synthesis. It was suggested that taurine enhanced LDL receptor activity

roxylase activity which catalyses the rate-limiting step in bile acid synthesis. The
ulation of 7α-hydroxylase activity increases bile acid production leading to
eased utilization of cellular cholesterol and enhanced LDL uptake. The increase
iliary cholesterol may be the consequence of increased concentration of bile
s, since bile acids function to keep the cholesterol in solution in the bile.
eased removal of cholesterol from the bile may result in releasing the feedback
bition of cholesterol on cholesterol synthesis, which may explain the observed
ease in cholesterogenesis.

-Alanine is an inhibitor of taurine transport into the cells. It competes with
ine for the specific transport site. The decrease in the concentration of taurine in
liver in the β-alanine treated rats is the result of this inhibition of transport of
ine. But the fact that both β-alanine and taurine treatments gave similar results
r as cholesterol metabolism is concerned indicates that β-alanine in the cells may
tion in the same manner as taurine. This is understandable in view of the close
ctural similarity of the two substances, the only difference being the presence of
OH in β-alanine in place of the SO_3H group in taurine. It is quite possible that
anine may also from conjugates like taurine with bile acids.

he fact that liver total cholesterol is not significantly altered in rats treated with
anine and taurine may be because the increased rate of its degradation to bile
s and removal of free cholesterol from the bile more than offset the increased rate
s synthesis.

vity of lipoprotein lipase in heart and adipose tissue

t β-alanine functions like taurine in the tissue is further indicated by the effect
the substances have on the activity of lipoprotein lipase (LPL). Enzyme activity
gnificantly increased in the heart by both the substances, and decreased in
ose tissue (table 5). LPL is responsible for the removal of circulating triglyceride-
lipoproteins by extrahepatic tissues. The increase in the concentration of tri-
erides in the heart in rats given β-alanine and taurine may be a result of the
ease in the activity of LPL.

Table 5. Effect of administration of β-alanine and taurine on activity
of LPL in the heart and adipose tissue of rat.

Tissue	LPL activity (μmol glycerol/h/g protein)		
	Group 1	Group 2	Group 3
Heart	34.8 ± 0.76	45.1 ± 1.13^a	53.2 ± 1.34^a
Adipose tissue	129.0 ± 3.50	86.5 ± 2.08^a	60.6 ± 1.40^a

Details as in table 1.

ease of lipoproteins into the circulation

Table 3. Effect of administration of β-alanine and taurine on release of lipoproteins into the circulation in rat.

| | Lipoprotein cholesterol (mg/100 ml serum) | | |
Group	Triton-injected group (A)	Saline-injected group (B)	Per cent difference (A–B)*
1	165.8 ± 4.15	81.8 ± 2.13	102.7 ± 2.67
2	109.5 ± 2.41	97.5 ± 2.44	12.3 ± 0.31^a
3	88.4 ± 1.86	83.9 ± 1.93	5.3 ± 0.13^a

*Difference (A–B) gives lipoprotein cholesterol released into the circulation. See text (materials and methods) for details. Other details as in table 1.

may be mostly channelled for bile acid synthesis rather than for lipoprotein synthesis.

These results indicate that though β-alanine reduces the level of taurine in the tissue, it replaces taurine in the cells functionally as far as the effect on cholesterol metabolism is concerned. It has been reported that taurine administration increases the activity of LDL receptors (Stephen *et al.*, 1987). The effect of β-alanine in this aspect was not studied. Thus, producing taurine deficiency by β-alanine does not appear to be useful for studying the metabolic role of taurine.

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Lipid peroxidation of hyperlipemic rat serum lipoproteins in chronic ethanol and acetaldehyde administration

RAMESH CHANDER, NARINDER K. KAPOOR* and
CHANAN SINGH

Division of Biochemistry, Central Drug Research Institute, Lucknow 226 001, India

Abstract. The levels of lipid peroxides in circulatory lipoproteins increased with chronic administration of ethanol or acetaldehyde. Low density lipoprotein showed a greater increase in its content of lipid peroxides than very low density lipoprotein or high density lipoprotein. However, very low density lipoprotein was more prone to lipid peroxidation *in vitro* than low density lipoprotein or high density lipoprotein. The effect of acetaldehyde was more marked than that of ethanol. Lipoproteins of control and hyperlipemic groups were partially protected against peroxidation by butyrated hydroxytoluene and serum high density lipoprotein of normal rats.

Keywords. Lipid peroxidation; serum lipoproteins; hyperlipemia; alcoholism; high density lipoprotein; butyrated hydroxytoluene.

Introduction

The chronic administration of ethanol or acetaldehyde is known to increase the levels of serum lipoproteins and causes the emergence of an abnormal lipoprotein, lipoprotein-X (Chander *et al.*, 1987). Increased levels of serum lipid peroxide (LPO) were found in chronic alcoholism (Fink *et al.*, 1985). β-Lipoproteins (very low density lipoprotein, VLDL; and low density lipoprotein, LDL) and the process of lipid peroxidation in general play an important role in the pathogenesis of coronary vascular diseases and atherosclerosis (Morel *et al.*, 1983; Mizukami *et al.*, 1984), which are also known to be associated with alcoholism. However, information on lipid peroxidation of lipoproteins in alcoholism and the interrelationship among lipoproteins in this respect is hardly available in the literature. Since high density lipoprotein (HDL) is known to give protection against cytotoxicity of β-lipoproteins *in vitro* (Hessler *et al.*, 1979), it was considered of interest to ascertain if HDL could afford protection against lipid peroxidation of β-lipoproteins during chronic administration of ethanol or acetaldehyde in rats. The studies described in this paper demonstrate partial protection from lipid peroxidation of β-lipoproteins by HDL from normal rats.

Materials and methods

Heparin and dextran sulphate (molecular weight 500,000) were purchased from Loba-Chemie, Vienna, Austria, and Sigma Chemical Co., St. Louis, Missouri, USA,

respectively. Butyrated hydroxytoluene (BHT) and other chemicals used were of analytical grade.

Male adult rats of Charles Foster strain (150–200 g) inbred in the CDRI animal house were divided into 3 groups of 8 rats each. They were administered normal saline, 50% aqueous ethanol (3·76 g/kg body weight) and 20% acetaldehyde (1·3 g/kg body weight), respectively by gastric tubing once a day for 60 days. At the end of 20, 40 and 60 days of alcohol/acetaldehyde treatment, animals were taken from each group. The animals were fasted overnight, blood was withdrawn by retro-orbital plexus and the animals sacrificed to collect the liver. The serum was fractionated into VLDL, LDL and HDL by the polyanionic precipitation method (Burstein et al., 1982) using heparin, dextran sulphate and $MnCl_2$ as reactants. Each fraction was dialysed against 0·1 M NaCl containing 0·05% EDTA in the presence of N_2 gas. LPO content of liver and serum lipoproteins was estimated by the thiobarbituric acid reaction (Ohkawa and Ohishi, 1978). Protein was estimated according to the method of Lowry et al. (1951). Lipid peroxidation of VLDL and LDL and protection by normal HDL were studied in vitro according to Hessler et al. (1979). Serum lipoproteins (100–200 μg protein) of control as well as hyperlipemic rats were mixed with normal rat serum HDL (N–HDL) solution (250–500 μg protein). The same amounts of VLDL and LDL were mixed with 6–10 μl (containing 6–10 pmol) BHT. A set of control tubes without addition of N–HDL or BHT was also prepared. LPO content in all these sets were estimated at zero time as well as after incubation for 6 h at 37°C. Protection against lipid peroxidation was calculated by comparison of LPO levels at zero time and 6 h.

Results

The effects of chronic administration of ethanol and acetaldehyde on LPO levels of serum lipoproteins and liver are given in table 1. It may be seen that LPO levels in

Table 1. LPO* of serum lipoproteins and liver in hyperlipemic rats.

Serum or tissue	Experimental schedule	Period of treatment (days)		
		20	40	60
Total serum	Control	$32·30 \pm 2·80$	$33·30 \pm 2·50$	$35·31 \pm 3·15$
	Ethanol-fed	$34·36 \pm 3·45^b$	$46·56 \pm 4·60$	$58·97 \pm 5·00$
	Acetaldehyde-fed	$36·18 \pm 3·00^a$	$57·26 \pm 4·00$	$61·43 \pm 4·94$
Serum-VLDL	Control	$70·25 \pm 4·27$	$67·94 \pm 3·75$	$72·63 \pm 4·20$
	Ethanol-fed	$78·45 \pm 4·90^a$	$84·74 \pm 3·40$	$92·13 \pm 3·85$
	Acetaldehyde-fed	$81·71 \pm 5·71$	$90·30 \pm 4·26$	$102·5 \pm 5·23$
Serum-LDL	Control	$116·4 \ \pm 12·5$	$117·40 \pm 10·20$	$123·30 \pm 10·20$
	Ethanol-fed	$123·70 \pm 14·3^b$	$162·20 \pm 17·50$	$199·10 \pm 18·30$
	Acetaldehyde-fed	$137·60 \pm \ 5·90$	$188·20 \pm 14·40$	$218·50 \pm 10·00$
Serum-HDL	Control	$126·10 \pm 08·8$	$131·50 \pm 12·30$	$133·20 \pm 13·80$
	Ethanol-fed	$142·50 \pm 12·3^a$	$156·40 \pm 14·30$	$143·70 \pm 18·30^b$
	Acetaldehyde-fed	$145·10 \pm 11·40$	$158·80 \pm 05·80$	$163·50 \pm 07·20$
Liver	Control	$116·20 \pm 12·20$	$122·70 \pm 10·50$	$125·80 \pm 15·20$
	Ethanol-fed	$121·50 \pm 08·50^a$	$134·00 \pm 06·00^a$	$164·60 \pm 12·50$

control rats did not show any perceptible change during the experimental period of 60 days. The level of LPO was highest in case of HDL than in VLDL and LDL. The levels of LPO in total serum and the 3 lipoprotein fractions showed progressive increase in both treatments. LDL showed a greater increase in its content of LPO than VLDL and HDL in both alcohol and acetaldehyde-treated groups. Similarly LPO levels in liver in both groups also exhibited a progressive increase with ethanol or acetaldehyde treatment. The effect of acetaldehyde was more marked than that of ethanol in all cases.

Lipid peroxidation *in vitro* was examined in the serum and in serum lipoproteins after treatment of rats with ethanol and acetaldehyde for 60 days. The results (figures 1–4) show that VLDL was more prone to lipid peroxidation than LDL or HDL, and lipid peroxidation was higher in the acetaldehyde-fed group than in the ethanol-fed groups. Lipid peroxidation of lipoproteins was found to be partially inhibited by N–HDL and BHT. VLDL and LDL of control and hyperlipemic groups were protected against lipid peroxidation *in vitro* by BHT and N–HDL, the former being a more potent protector. VLDL was protected by BHT or N–HDL to a greater extent than LDL.
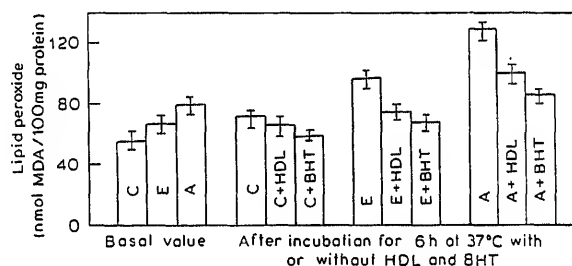
Figure 1. Protection from lipid peroxidation of hyperlipemic rat serum by normal HDL or BHT.
C, Control; E, ethanol-fed; A, acetaldehyde-fed.
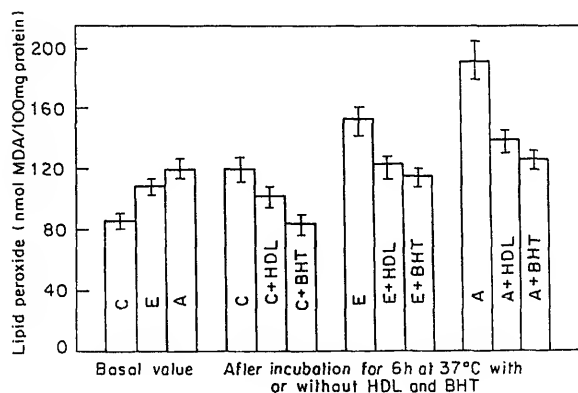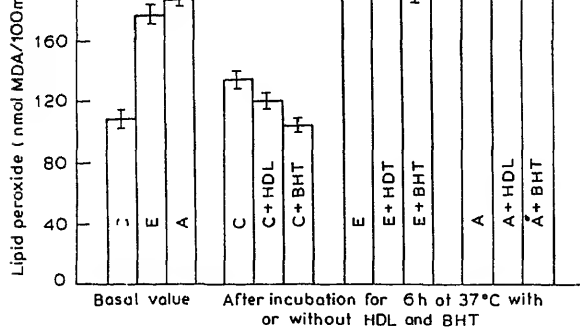
Figure 3. Protection from lipid peroxidation of hyperlipemic rat serum LDL by normal HDL or BHT.

C, Control; E, ethanol-fed; A, acetaldehyde-fed.
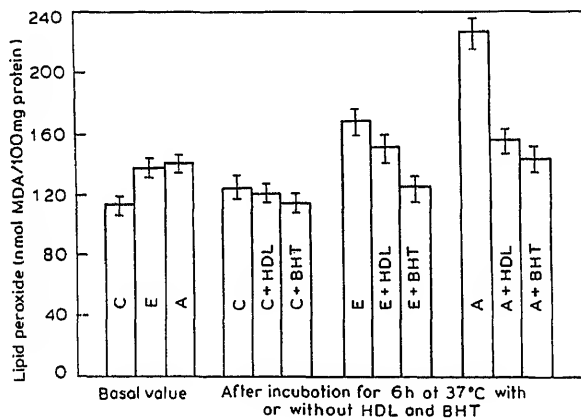
Figure 4. Protection from lipid peroxidation of hyperlipemic rat serum HDL by normal HDL or BHT.

C, Control; E, ethanol-fed, A, acetaldehyde-fed.

Discussion

The investigation showed enhancement in LPO content of serum lipoproteins and liver under conditions of chronic administration of ethanol and acetaldehyde. The role of antioxidants in modifying hepatic injury and hyperlipemia induced by chronic feeding of ethanol would suggest that the primary events in the development of fatty liver and the damage undergone by it consist in the formation of LPO at selective subcellular sites (Di Luzio, 1973; Harta *et al.*, 1983). Since liver is a major site of synthesis of lipoproteins, hepatic injury could be accompanied by abnormalities of lipoprotein biosynthesis and metabolism which may be reflected in the blood lipoprotein spectrum (Vadivelu and Ramakrishnan, 1986). Increased

...toxic to cells and tissues. Ross and Harker (1976) reported that during ...erlipemia, lipoproteins may initiate and maintain atheromatous lesions by ...othelial cell injury and lipid accumulation. Recently it has been emphasised that ...erlipemic β-lipoproteins are cytotoxic to cells and tissues presumably due to ...anced levels of associated LPO (Jurgens et al., 1986). Hypertriglyceridemic-...DL and LDL are known to suppress the activity of 3-hydroxy-3-methylglutaryl ...nzyme A reductase in cultured fibroblasts from human subjects; they also exert ...c effects on endothelial cells (Gianturco et al., 1980).

...ur findings indicate that BHT (an antioxidant) as well as N–HDL provide ...ection to VLDL and LDL in vitro against lipid peroxidation. These results ...ld seem to be consistent with recent epidemiological data linking the high LDL ... low HDL concentration in circulation with the development of accelerated ...rosclerosis (Narula and Wasir, 1985). The property of HDL to act as an ...oxidant for VLDL and LDL may mean that the cytotoxicity of pathogenic β-...proteins as observed in atherosclerosis and vascular diseases is due to their ...eptibility to lipid peroxidation (Henriksen et al., 1979; Evensen et al., 1983). ...taldehyde caused more pronounced lipid peroxidation than ethanol, which may ...ttributed to the fact that acetaldehyde is the immediate active metabolite of ...nol (Ramakrishnan 1984). Lipid peroxidation in alcoholism may augment the ...cts of atherosclerosis and vascular abnormalities. It seems that HDL plays a ...l role in the inhibition of free radical-induced lipid peroxidation of lipids and ...proteins.

...n conclusion it may be added that apart from other measures which are ...erally adopted against the pathophysiology/ill-effects of alcoholism, the use of ...oxidants, preferably of natural origin, viz., β-carotene, α-tocopherol, mannitol ... ascorbic acid, as an additional measure is strongly indicated (Morgan, 1982). ...h a protective measure may also prove to be useful in preventing atherosclerosis ... other vascular diseases.

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specific compartmental analysis of gonadotropin stimulation of ornithine decarboxylase

USHA NATRAJ*

Reproductive Endocrinology Program, Department of Pathology, University of Michigan, Ann Arbor, Michigan 48109, USA

*Present Address: Institute for Research in Reproduction, Jehangir Merwanji Street, Parel, Bombay 400 012, India

Abstract. Luteinizing hormone is known to stimulate the enzyme ornithine decarboxylase in the ovary. Highly purified human follicle stimulating hormone that is devoid of significant biologically active luteinizing hormone can also induce ornithine decarboxylase activity in intact immature rats with a time course of induction similar to that reported for luteinizing hormone. A maximum of 8–10-fold stimulation above controls was observed 4 h following intravenous administration of human follicle stimulating hormone. This stimulation followed a strict dose response relationship. Ovine luteinizing hormone and human chorionic gonadotropin always induced more ovarian ornithine decarboxylase activity than that achieved by maximally effective doses of follicle stimulating hormone. This could not be attributed solely to the ability of specific cell population to respond to the respective gonadotropins. Although granulosa cells contained little receptor for luteinizing hormone/human chorionic gonadotropin and the residual tissue contained little receptor for follicle stimulating hormone, each tissue responded to these gonadotropins in a manner suggestive of the mediation by one or more diffusable factors. A relationship between gonadotropin induced 3'5'-cyclic adenosine monophosphate (cyclic adenosine monophosphate) concentration and ornithine decarboxylase activity suggests that the mediation of gonadotropin stimulated ovarian ornithine decarboxylase is not solely through cyclic adenosine monophosphate, indicating the presence of other factors in the induction of gonadotropin increased ornithine decarboxylase activity.

Keywords. Ovarian ornithine decarboxylase; ovarian compartment; receptors; cAMP; gonadotropins.

ction

alian ornithine decarboxylase (ODC) (EC 4·1·1·17. L-ornithine carboxylyase), es the decarboxylation of L-ornithine to putrescine and carbon dioxide. This first and rate limiting reaction in polyamine biosynthesis (Morris and ame, 1974).

anced elaboration of polyamines associated with increased macromolecular esis has been repeatedly observed. A large and rapid increase in ODC activity aracteristic early event in several hormone stimulated target tissues, including n ODC stimulated by human chorionic gonadotropin (hCG) and luteinizing ne (LH) (Kaye *et al.*, 1973). Whether or not follicle stimulating hormone can stimulate ovarian ODC *in vivo* has not been completely resolved (Sheela nd Moudgal, 1979).

In this report, using highly purified human FSH (hFSH) with insignificant
mination of biologically active LH, we demonstrate that hFSH can stimulate
in a time and dose dependent manner in intact immature female rats and th
response depends to a large extent on the route of administration. Further, it a
that much greater consideration must be given to differential responsiver
ovarian compartments. Additional evidence indicates that gonadotropin stimu
of ODC may at least in part, involve a secondary intracellular mediator which
cAMP.

Materials and methods

Intact immature female rats obtained from Holtzman, Madison, Wisconsin
were maintained on pelleted food and water *ad libitum*.

hFSH, oLH and hCG (Roussel Corp., 2950 IU/mg) in 0.1% gelatin/(
phosphate buffer containing 0.14 M NaCl, pH 7 (PBS) were administered
subcutaneously or intravenously. 17-β-Estradiol in propylene glycol was admin
subcutaneously.

Pyridoxal 5′ phosphate (PLP), dithiothreitol (DTT), DL-ornithine and
estradiol were obtained from Sigma Chemicals Co., St. Louis, Missouri, USA. [1
Ornithine monohydrochloride (52.8 mCi/mmol) and hyamine hydroxide
obtained from New England Nuclear, Boston, USA. Glass tubes (16 × 100 mn
stoppers fitted with center wells were obtained from Kontes Glass Co., New
USA. All other chemicals used were of analytical grade.

Granulosa cell expression

The ovaries were trimmed of extra ovarian tissue and placed in cold PBS. Gra
cells were isolated in PBS by applying gentle pressure to the ovaries with a
spatula. The rest of the ovary was designated residual tissue (Zeleznik *et al.*,
This tissue contained theca, interstitial cells and non-expressed granulosa cel
expressed granulosa cells and the residual tissue were washed twice with F
remove follicular and interstitial fluid, respectively.

Preparation and assay of ODC

ODC activity was extracted and assayed according to Kaye *et al.* (1973). Brie
ovaries, granulosa cells or residual tissue were homogenized in a Potter-El
homogenizer in isotonic sucrose (0.25 M), containing 25 mM Tris-HCl (p
5 mM DTT, 0.1 mM EDTA and 1 μM PLP. The homogenate was centrifug
30 min at 30,000 g in a Sorvall refrigerated centrifuge. The supernatant was u
an enzyme source. The standard enzyme assay contained 50 mM Tris-HCl (p
0.1 mM PLP, 5 mM DTT, 0.5 mM DL-ornithine containing 0.2 μCi of lab

lts

e course of changes in ODC activity

nges in the activity of ODC in granulosa cells and residual tissue in response to a
le subcutaneous injection of oLH in intact immature rats are depicted in figure 1.
enzyme activity increased rapidly in both the granulosa cell and residual tissue
partment in response to 30 μg oLH. A maximum increase occurred at 4 h
wing hormone administration and then declined rapidly. On the basis of this
the results of replicate experiments, most subsequent studies were terminated at
When, 2 μg of FSH was administered subcutaneously and enzyme activity
sured in granulosa and residual tissues, no increase in enzyme activity was
rved.
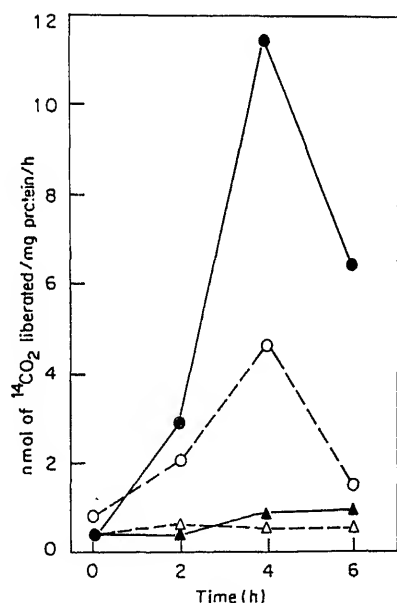
Figure 1. Time course of ovarian ODC stimulation in response to oLH and hFSH. 24 day
old intact immature rats were administered either a single injection of oLH (30 μg, sub-
cutaneously) or hFSH (2 μg, subcutaneously). The enzyme activity in the residual tissue and
dispersed granulosa cells were estimated as detailed in the text. (●), ODC activity in the
residual tissue; (○), granulosa in response to oLH; (▲), residual; (△), granulosa cell in
response to hFSH. Values are mean of two closely agreeing values.

ts of FSH, hCG, estradiol and FSH + estradiol ODC activity

re 2A depicts changes in ODC activity in whole ovaries of intact immature rats
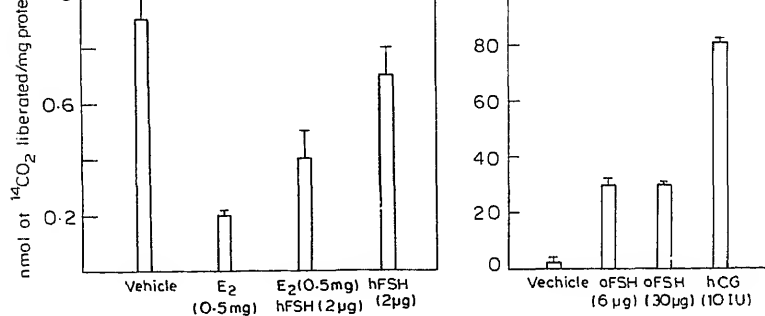
Figure 2. **A.** Levels of ovarian ODC in response to estrogen and hFSH. 24 day old intact immature rats were treated with various hormones in groups of 4. Animals were sacrificed 4 h following the hormone administration and enzyme activity measured as detailed in text. Values are expressed as mean ± SEM (n=4). **B.** Levels of ovarian ODC activity response to FSH/hCG. 24 day old intact immature rats received hormones by intravenous route and animals were sacrificed 4 h later and the enzyme activity measured. Values are expressed as mean ± SEM (n=4).

failed to stimulate ODC activity, while a single injection of 17-β-estradiol (500 decreased the basal activity. A combination of both these hormones also failed stimulate the enzyme activity. However, intravenous administration of oFSH hFSH to intact immature rats caused a dramatic increase in ovarian ODC activity (figure 2B). A 15-fold increase in activity was observed when 6 μg of oFSH w administered and a further 5-fold increase in this (30 μg) dose did not result in a further increase in the enzyme activity. When 10 IU of hCG was given intravenous enzyme activity increased to 3-fold higher than that obtained with a maxima effective dose of hFSH (6 μg).

Time course of ODC activity

The changes in response to intravenous hFSH with time are depicted in figure Intravenous injection of 5 μg hFSH stimulated ODC in a time dependent manner significant increase over the saline treated control occurred as early as 1 h follow the hormonal injection. Maximal stimulation occurred 4 h following hormo treatment. By 6 h the activity started to decline and enzyme activity could not distinguished from saline treated controls by 12 h.

Dose response relationships

The effects of increasing dosage of hFSH on ODC activity measured 4 h a intravenous administration are shown in figure 4. As little as 1 μg of hFSH increa
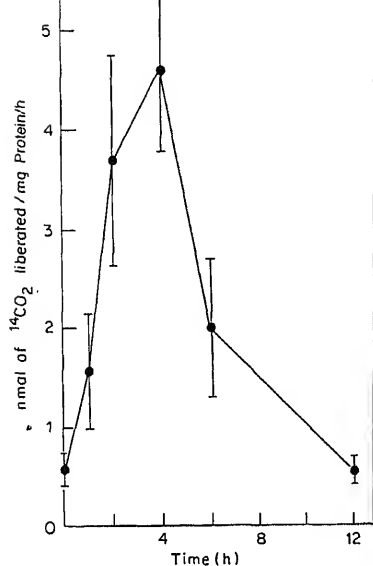
Figure 3. Time course of ovarian ODC stimulation by hFSH. Details are as described in the text.
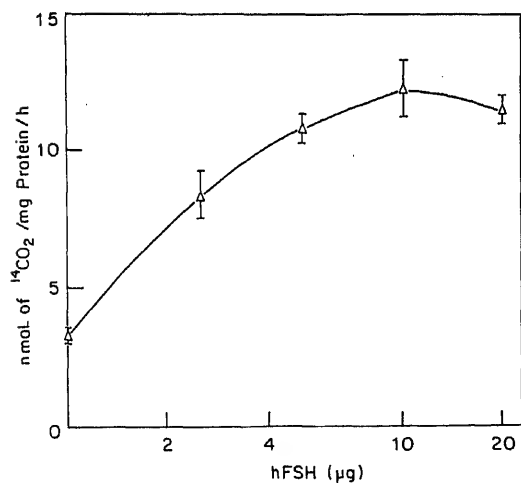
Figure 4. Dose dependent increase in ODC in response to hFSH. Animals were sacrificed 4 h following the hormone administration. Values are expressed as mean $\pm$ SEM (n = 4).

rtmentalization of gonadotropin stimulated ovarian ODC and cAMP

5 summarizes the results of measuring hCG/hFSH stimulated ODC activity
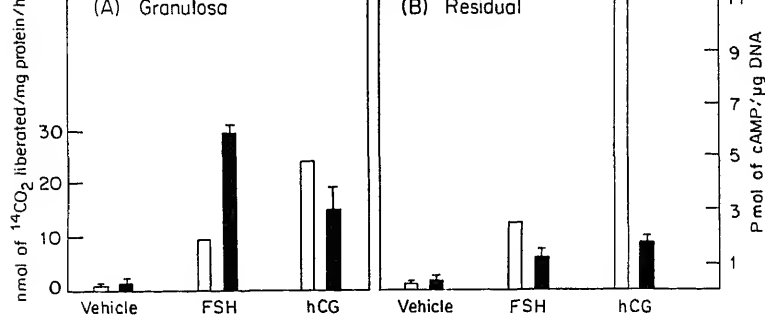
Figure 5. Gonadotropin stimulated ODC and cAMP in 24 day intact immature rats. ODC activity (□), and cAMP concentrations (■) were measured 4 and 1 h following hormonal administration (peak period) respectively. ODC activity was measured as detailed in the text. Values are mean of duplicates. cAMP values are mean ± SEM (n = 4).

pooled from 8 ovaries. The typical results are depicted in the figure 5. In untreated or saline treated control animals, the specific activity of ODC in granulosa cells was similar to that in residual tissue. The administration of 5 μg of hFSH caused a 6-fold stimulation in ODC activity in both the granulosa cells and residual tissue at 4 h. Under the same conditions, 10 IU of hCG increased the activity of the enzyme 17-fold in granulosa cells and 25-fold in the residual compartment. Concentrations of cAMP were measured at the time of maximum stimulation, 60 min following the hormone treatment. Following 5 μg of hFSH (iv) administration cAMP increased 14-fold over the control in the granulosa cells, but only 3-fold in the residual compartment. Administration of hCG (10 IU) also increased the concentration of cAMP 7-fold in the granulosa cells and 4-fold in the residual tissue.

Effects of db-cAMP and theophylline on ODC activity

Table 1 summarizes the stimulation of ovarian ODC activity obtained with combined db-cAMP and theophylline, a phosphodiesterase inhibitor. Theophylline alone had no effect on basal enzyme activity. Simultaneous injection of db-cAMP and theophylline (1 mg) stimulated ODC. However, the increase was only 3-fold over the control levels. Higher doses of db-cAMP and 1-methyl-3-isobutyl-xanthine, another phosphodiesterase inhibitor or theophylline were toxic to the animals.

Table 1. Stimulation of ovarian ODC in 24 day old intact rats by theophylline, dibutryl cAMP and hCG.

Treatment	ODC activity (n mol of $^{14}CO_2$ liberated/mg protein/h)
Vehicle	2 ± 0.2
hCG (10 IU, iv)	99 ± 8.5
db-cAMP (5 mg) + theophylline (1 mg)	6.2 ± 2.3

2 depicts the specific binding of ^{125}I-hCG or ^{125}I-FSH to the granulosa cells
dual tissue used for ODC assay. Granulosa cells contained most of the FSH
or, while residual tissue contained less than 10% of the total FSH receptor.
rsely, residual tissue contained 92% of the hCG receptor, while granulosa cells
ned only 8% of the total hCG receptor activity. This distribution of gonado-
receptor activity agrees well with previous reports (Richards, 1979).

Table 2. Distribution of gonadotropin receptor in the ovary
of intact immature rats.

	cpm/μg DNA		Ratio R/G
	Granulosa	Residual	
^{125}I-FSH	652 ± 77	$62 \cdot 5 \pm 7 \cdot 5$	0·0958
^{125}I-hCG	$65 \cdot 5 \pm 12$	$805 \cdot 25 \pm 25$	12·29

For the measurement of binding, ^{125}I-labelled hCG and ^{125}I-labelled hFSH, were used under saturating conditions in the presence and absence of excess unlabelled hormone. The difference represents specific binding. Values are mean ± SEM (n = 4).

sion

hed results indicate that FSH is not an inducer of ODC in the mammalian
in vivo (Osterman and Hammond, 1977; Sheela Rani and Moudgal, 1979;
lis *et al.*, 1981; White and Ojeda, 1981). In accord with this we found that a
subcutaneous injection of purified FSH, that had been treated with chymo-
1 to remove LH contamination, failed to stimulate ovarian ODC when
red 1, 2, 4 or 6 h following the hormone administration (figures 1, 2A). This
des a shift in the time course of enzyme stimulation. The dose of hFSH used
least twice the amount needed to induce and promote near maximal follicular
pment and to increase the incorporation of tritiated thymidine into DNA (Rao
1978). A combination of estradiol and FSH also failed to increase ODC.
ver, a single intravenous administration of FSH increased the ovarian ODC
y in a time and dose dependent manner. The ODC response followed a strict
ourse and peaked at 4 h, similar to the results observed with other systems
et al., 1973). The response showed dose dependency over the range of 1–10 μg.
the inability of other investigators to demonstrate that FSH can stimulate
n ODC *in vivo*, may be due to the use of the subcutaneous route with an in-
ent dose. Although plasma concentrations of FSH after injection by these two
were not compared in our study, it is certain that higher concentrations would
been reached following intravenous administration at least shortly after
on. This is the most likely explanation of the results, and suggests that FSH

no LH responsive adenylate cyclase (Richards *et al.*, 1979). It was surprising to
that administration of LH to immature rats with preantral follicles led to ma
increase in ODC activity in isolated granulosa cells (figure 1). To understand
results more fully, we attempted to determine which of the following altern
explanations could be responsible for the increased activity: (i) stimulation
contaminant, most likely FSH, (ii) contamination of granulosa cells by co-expre
LH-responsive cells, (iii) indirect stimulation by a product derived from
responsive cells and (iv) unusual responsivity of granulosa cell ODC activity to
stimulation.

The first possibility, that the stimulation of ODC in granulosa cells from prea
follicles was due to a contaminant, most likely FSH, seems unlikely. The oLH us
these studies is a highly purified preparation (LER 1733, $1.64 \times$ NIH-LH-Sl.
contamination $0.04 \times$ NIH-FSH-Sl). Thus, the amount of contaminating
injected with the oLH was far less than that found necessary for inducing (
activity. Further, hCG also stimulated ODC activity in granulosa cells
preantral follicles and this hormone is relatively free of FSH activity.

The second possibility that expressed granulosa cells are contaminated by
expressed LH-responsive cells, can almost be discarded on the basis of the bir
studies. Thus, the specific activity of LH/hCG receptor sites in the expressed g
losa cell preparation considered on a per cell basis (CPM/μg DNA) was only 8
the activity in the residual tissue. For this explanation to be valid, the contamin
LH/hCG receptor positive cells should have an ODC inducible system that is a
7 times more responsive to hCG than the ODC responsive system present in th
of the residual cells (figure 5).

Data are not at hand to rule out either of the last two possibilities. LH/
receptor activity present in the granulosa cell preparation may be associated w
sub-population of cells that are unusually responsive to receptor site occupatio
hCG. The possibility that the granulosa cells are actually being stimulated indir
by an extra-cellular factor derived from hCG responsive cells, also can not be
out. Prostaglandins, catacholamines, polyamines, cyclic nucleotides and
peptide factors in follicular fluid are all candidates for such a mediator. The la
response to very large amounts of estradiol does make this steroid an unl
candidate.

The apparent lack of correlation amongst gonadotropin receptor distribu
ODC and cAMP activities in granulosa and residual tissue, respectively,
suggests differences in the coupling efficiency of hormone receptor and aden
cyclase activation in these two compartments.

The submaximal doses of hCG and oLH used in this study were more effecti
stimulating ODC activity than all the doses of FSH tested including those that w
were maximally effective. This was true for the whole ovary, the granulosa cells
the residual tissue. In spite of this, FSH was more effective than hCG in stimul
granulosa cell accumulation of cAMP (figure 5). These relative effects on cAMF
ODC are extremely difficult to reconcile with the postulates that cAMP might a
the exclusive second messenger for gonadotropin stimulation of ODC (Johnson
Sashida, 1977; Osterman *et al.*, 1978). Indeed, the results support the suggestion

involved remains to be identified.

Acknowledgements

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Mechanism of down regulation of luteinizing hormone receptors and steroidogenesis in corpora lutea

RAJAN R. DIGHE and N. R. MOUDGAL

Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India

Abstract. The mechanism of 'down regulation' of luteinizing hormone receptors was investigated in pseudopregnant rats using a modified radioimmunoassay capable of measuring endogenous tissue-bound hormone. Treatment of pseudopregnant animals with a desensitizing dose (desensitization treatment) of human chorionic gonadotropin resulted in a decrease in receptor concentration. This decrease was prevented if the animals were treated prior to the desensitization treatment with indomethacin, an inhibitor of prostaglandin biosynthesis, suggesting a role for prostaglandins in down regulation. The desensitization treatment resulted in a time-dependent decrease in subsequent responsiveness of the tissue to luteinizing hormone. Basal progesterone production rate was also decreased following desensitization. Total tissue cholesterol was found to be decreased following desensitization treatment, without any change in the ratio of free to esterified cholesterol. Mitochondrial cholesterol was significantly reduced and pregnenolone production by the mitochondria of desensitized corpora lutea was also markedly reduced. However, when cholesterol was added to the mitochondria of desensitized corpora lutea, pregnenolone production was increased, reaching values almost equal to that shown by the control mitochondria. These results show that decrease in the responsiveness following desensitization treatment is due to, besides receptor loss, decrease in tissue cholesterol, in particular mitochondrial cholesterol. The cholesterol side chain cleavage activity, although low, appears to be functionally intact; the low activity could be attributed to low levels of mitochondrial cholesterol.

Keywords. Down regulation; desensitization; luteinizing hormone; steroidogenesis.

Introduction

It is a well-recognized property of several hormones and drugs that the primary stimulus modulates the responsiveness of the target tissue to subsequent exposure of the same tissue to the same hormone. This phenomenon is known as 'desensitization' or 'down regulation' and has been demonstrated for several hormones and growth factors. Administration of luteinizing hormone (LH) or human chorionic gonadotropin (hCG) has been shown to cause decrease in LH receptors both in ovaries and testes (Dufau and Catt, 1979). Such a decrease in receptors is also followed by decrease in responsiveness of the tissue to the same stimulus (Dufau and Catt, 1979). It was further demonstrated that steroidogenesis in desensitized tissue could not be stimulated by dibutyryl cAMP or cholera toxin, suggesting a defective steroidogenic pathway (Conti et al., 1977). However, the mechanism by which the hormones cause decrease in the receptors and alter the responsiveness of the tissue is poorly understood. In the present study an attempt has been made to unravel the

Materials and methods

Hormones and chemicals

hCG used in this study was a kind gift of Dr C. R. Canfield, USA. Ovine LH (o...
was a gift of Dr M. R. Sairam, Canada. Pregnant mare serum gonadotropin (PM...
was purchased from Sigma Chemical Co., St. Louis, Missouri, USA. [^{3}H]Pro...
terone, [^{3}H]pregnenolone and Na^{125}I were purchased from the Radiochem...
Centre, Amersham, UK. All reagents used in this study were of analytical grade.

Animals

Immature female rats 25–26 days of age were rendered pseudopregnant by inject...
subcutaneously 15 IU of PMSG followed 56 h later by 50 IU of hCG. Four to...
days after hCG treatment the animals were treated with either saline o...
desensitizing dose of hCG (referred to hereafter as 'desensitization treatment')...
killed after various times depending on the experimental design. Ovaries...
removed, and corpora lutea were dissected free of adhering fat using a fine ne...
and processed according to different experimental designs.

Radioimmunoassay of tissue-bound hCG

Luteal tissue-bound hCG was determined essentially according to the proced...
previously used for tissue-bound LH (Muralidhar and Moudgal, 1976a, b) and tis...
bound follicle stimulating hormone (Sheela Rani and Moudgal, 1978). Corpora l...
were homogenized in 0·05 M Tris–HCl, 0·05 M EDTA, pH 7·4, and different aliq...
were used for assaying hCG in the tissue by radioimmunoassay (RIA) carried ou...
37°C as described by Muralidhar and Moudgal (1976a, b).

Determination of responsiveness of corpora lutea

Responsiveness of the corpora lutea from control and hCG-treated animals to...
was determined by incubating the corpora lutea in Krebs–Ringer bicarbo...
medium, pH 7·4, containing 0·05 M Hepes and 0·1% bovine serum albumin (BS...
the absence or presence of LH (0·1, 1 and 10 μg/ml oLH) for 2 h at 37°C. Pro...
terone secreted into the medium was estimated by a specific RIA.

Determination of luteal tissue cholesterol

Corpora lutea were obtained from animals killed 12 or 24 h after desensitiza...
treatment. The corpora lutea from individual animals were suspended in saline...
stored frozen until further use. To determine luteal tissue cholesterol and ester...
cholesterol, corpora lutea were homogenized in 0·15 M NaCl and total lipids...

Determination of cholesterol side chain cleavage activity

Cholesterol side chain cleavage (SSC) activity of the mitochondria-rich preparation was assayed by estimating pregnenolone produced by the mitochondria. Corpora lutea were homogenized in 0·05 M Tris–HCl, pH 7·4, containing 0·5 mM EDTA and 0·25 M sucrose. The homogenate was spun at 600 g for 10 min. The supernatant was retrieved and centrifuged at 8500 g for 15 min. The pellet was resuspended in the same buffer after washing and used for determining SSC activity. Aliquots of mitochondria (approximately 300 μg protein) were incubated in a total volume of 1 ml of a solution containing 5 mM Ca^{2+}, 5 mM Mg^{2+}, 40 mM nicotinamide, and 10 mM sodium succinate. Cholesterol (100 μM), whenever present, was added in 50 μl absolute ethanol; all other aliquots received the same volume of ethanol. The reaction was initiated by adding the mitochondrial preparation and was continued for 30 min at 37°C. The reaction was terminated by placing the tubes in a boiling water bath for 1 min. Steroids formed were extracted with ether, and separated by thin-layer chromatography using chloroform:ethyl acetate (4:1) as the solvent system. Using this system, it was possible to separate pregnenolone from progesterone. Pregnenolone was then estimated by RIA using 7-[^{3}H]–pregnenolone as the tracer and a progesterone antiserum with 100% cross-reactivity with pregnenolone.

Results

Demonstration of down regulation of LH receptors

Pseudopregnant rats were treated subcutaneously with saline or a desensitizing dose of hCG (4 μg). The animals were again treated after 48 h with hCG (2 or 4 μg) and killed 3 h later. Tissue-bound hCG was determined by RIA. It was earlier found that maximum uptake of hCG by the ovaries occurs 3 h after a subcutaneous injection of hCG and the uptake was maximal when 4 μg of hCG were administered, suggesting saturation of LH receptors *in vivo*. As shown in table 1, animals that had received desensitization treatment showed a significant decrease in uptake of hCG, demonstrating down regulation of LH receptors following desensitization treatment.

Involvement of prostaglandins in down regulation of LH receptors

The involvement of prostaglandins in the process of down regulation of LH

Table 1. Down regulation of LH receptors.

Group	hCG (μg) treatment at 0 h	48 h	Luteal tissue hCG (ng/10 mg tissue)*
A	—	—	0·05 ± 0·11
B		2	12·00 ± 2·0

inhibitor on hCG-induced receptor loss. The animals were administered 1 and 4 μg of hCG as a desensitization treatment and uptake of hCG 3 h following the administration of 5 μg of hCG 48 h after desensitization was determined. One group of animals was treated with 500 μg indomethacin 1 h prior to desensitization treatment and hCG uptake by luteal tissue was determined as described above. As shown in table 2, the desensitizaton treatment resulted in a dose-dependent decrease in hCG

Table 2. Effect of indomethacin on hCG-induced receptor loss.

Group	Treatment at −1 h Indometha-cin (μg)	Treatment at 0 h hCG (μg)	Treatment at 48 h hCG (μg)	Luteal tissue hCG (ng/10 mg tissue)*
A	—	—	5	9.5 ± 1.5
B	—	4	—	0.45 ± 0.05
C	—	4	5	2.15 ± 0.18
D	500	4	—	0.38 ± 0.05
E	500	4	5	2.9 ± 0.13
F	—	1	—	ND
G	—	1	5	3.5 ± 0.3
H	500	1	5	8.6 ± 1.45

*Values are mean ± SD, n = 4.
Significance of difference: C vs A, $P < 0.001$; G vs A, $P < 0.001$; E vs C, $P < 0.005$; H vs G, $P < 0.005$.
ND, Not determined.

uptake by the corpora lutea, suggesting loss of LH receptors. Indomethacin treatment reduced receptor loss. This effect was only marginal when the desensitizing dose was 4 μg but completely prevented receptor loss when the desensitizing dose was 1 μg.

Effect of desensitization treatment on responsiveness of the corpora lutea

Corpora lutea obtained from rats after various times following desensitization treatment were incubated with different concentrations of oLH or without oLH for 2 h at 37°C. Progesterone secreted into the medium was estimated by RIA. As shown in figure 1, the control corpora lutea (0 h) responded to LH *in vitro* by producing increased progesterone. However, the corpora lutea obtained 4, 12, 24 and 48 h after the desensitizing dose did not show any responsiveness to LH added *in vitro*. The corpora lutea obtained 4 h after desensitizing treatment showed increased basal production *in vitro* but did not show any further responsiveness. Corpora lutea obtained 12, 24 and even 48 h after the desensitization treatment showed decreased basal production of progesterone.

Effect of desensitization treatment on sterol levels of luteal tissue
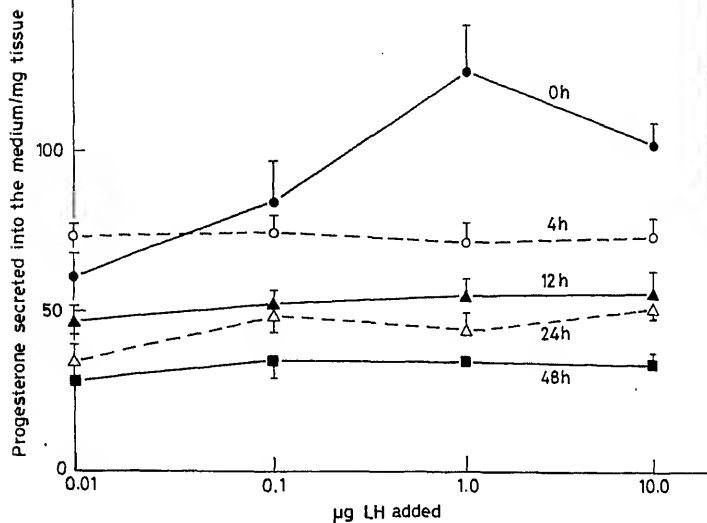
Figure 1. *In vitro* responsiveness of the corpora lutea following a desensitization treatment. Corpora lutea were obtained from animals 4, 12, 24 and 48 h after treatment; 0 h indicates untreated animals (control). Each point is the mean ±SD of triplicates.

controls. As shown in table 3 there was a significant decrease in cholesterol levels ($P<0.05$), although by 24 h the cholesterol levels appear to be higher than at 12 h

Table 3. Endogenous free and esterified cholesterol in control and desensitized corpora lutea.

Time after hCG treatment (h)	Cholesterol (μg/mg tissue)	Esterified cholesterol as cholesterol equivalent (μg/mg tissue)	Total cholesterol* (μg/mg tissue)
0	0·99 ± 0·13	2·36 ± 0·24	3·37 ± 0·37
12	0·63 ± 0·12	1·54 ± 0·36	2·46 ± 0·40
24	0·73 ± 0·14	1·88 ± 0·29	2·61 ± 0·40

*Values are mean ± SD, n = 4.
Significance of difference: Cholesterol 12 h *vs* 0 h, $P<0.05$; cholesterol 24 h *vs* 0 h, not significant; cholesterol ester, 12 h *vs* 0 h, $P<0.05$; cholesterol ester 24 h *vs* 0 h, not significant; total cholesterol 12 h *vs* 0 h, $P\simeq0.05$.

and not significantly different from 0 h levels. A similar pattern was observed for esterified cholesterol, and for free and esterified cholesterol taken together (total cholesterol). However, total cholesterol present in the mitochondria obtained from pooled corpora lutea was markedly reduced even 24 h after the desensitization treatment (table 4).

Effect of desensitization treatment on cholesterol side chain cleavage activity

dria-rich preparation obtained from
control and desensitized corpora lutea.

Treatment	Mitochondrial cholesterol (μg/mg protein)
Saline	27·6
hCG (4 μg)	13·95

Animals were killed 24 h after treatment.

activity. As shown in table 5, pregnenolone production by mitochondria from hCG-treated rats was significantly lower than that by control mitochondria. While addition of exogenous cholesterol to mitochondria from control corpora lutea did

Table 5. Cholesterol side chain cleavage activity in control and desensitized luteal mitochondria.

Group	Pregnenolone formed (pmol/mg protein)*
A Control luteal mitochondria	205·9 ± 37·4
B Desensitized luteal mitochon-dria	67·4 ± 20·4
C Control luteal mitochondria + cholesterol	180·1 ± 40·0
D Desensitized luteal mitochon-dria + cholesterol	200·1 ± 51·6

Animals were killed 24 h after desensitization treatment or saline injection (control).
*Values are mean ± SD, n = 4.
Significance of difference: B vs A, $P < 0.001$; D vs B, $P < 0.025$; C vs D, not significant.

not enhance pregnenolone formation, the mitochondria from desensitized luteal tissue showed stimulated production of pregnenolone and this was equal to that produced by the control mitochondria.

Discussion

In the studies presented above an attempt has been made to understand the mechanism of down regulation of LH receptors and the subsequent reduction in responsiveness of the corpora lutea. The down regulation of receptors was demonstrated using an RIA system extensively validated earlier (Muralidhar and Moudgal, 1976a, b; Sheela Rani and Moudgal, 1978; Dighe, 1982) for measurement of physiologically active LH bound to receptors. With this assay system it has been demonstrated here that decrease in hormone uptake following desensitization treatment was indeed due to a true loss of the receptors and not due to decrease in

twar, 1975; Zor and Lamprecht, 1977; Behrman, 1979). Prostaglandins have
en shown to cause reduction in LH receptor concentration (Hichens et al.,
rinwich et al., 1976a, b; Behrman and Hichens, 1976; Behrman et al., 1978). In
sent study it has been shown that inhibition of prostaglandin biosynthesis by
thacin treatment prevented hCG-induced receptor loss. The protection
d by indomethacin was dependent on the dose of indomethacin, 500 μg being
st effective dose (data not shown). At this dose inhibition of receptor loss was
if the desensitizing dose of hCG was 4 μg but complete inhibition of receptor
s observed when the desensitization dose of hCG was reduced to 1 μg. These
provide confirmatory evidence for the involvement of prostaglandins in
ng luteal cell LH receptor concentration. One of the events that occur
g binding of the hormone to the receptor is perhaps stimulation of prosta-
biosynthesis which in turn initiates down regulation of receptor. Haour et al.
b) have noted that in the Leydig cell prostaglandin levels increase 6 h following
dministration. In the present study it was also found that administration of
thacin 90 min or 6 h after desensitization treatment did not prevent the
r loss (data not shown). Thus receptor loss, once initiated, could not be
ed by indomethacin administration.

crease in the receptor concentration was also accompanied by decrease in
iveness of the corpora lutea. Progesterone production decreased in a time-
ent fashion and was reflected both in basal rate of progesterone production as
in response to LH in vitro. This suggested that in addition to receptor loss
nt alterations in the progesterone biosynthetic pathway should be occurring.
is a large body of evidence to show that loss of LH receptors is also
panied by several lesions in the steroid biosynthetic pathway. Luteal adenylyl
activity was found to be decreased following administration of a desensitizing
hCG to pseudopregnant rabbits (Hunzicker-Dunn and Birnbaumer, 1976).
, it was shown that desensitized Leydig cells responded to cholera toxin with
ed cAMP production, but failed to respond in terms of testosterone
tion (Tsuruhara et al., 1977). It has also been observed that desensitized luteal
not respond to cholera toxin and dibutyryl cAMP in terms of progesterone
tion (Conti et al., 1977). In the case of Leydig cells the lesion in testosterone
hesis was identified as a decrease in the activities of the enzymes 17–20 lyase
aga et al., 1978) and 17α hydroxylase (Chaslow et al., 1979). However, such
have not been identified in luteal cells. The levels of cholesterol and esterified
erol were found to be decreased in corpora lutea obtained from hCG-treated
. However it must be noted that there was no decrease in the ratio of free to
d cholesterol. Down regulation, as described here, results in decrease in
erone biosynthesis and this is similar to that occurring in corpora lutea
ing functional luteolysis. Blockade or reduction in endogenous LH
ility brought about by LH antibody treatment resulted in significant
lation of esterified cholesterol in the luteal tissue (Moudgal et al., 1972;
n et al., 1972; Mukku and Moudgal, 1980) and this was unlike that observed
ensitized corpora lutea. Corpora lutea undergoing luteolysis remained
ive to added LH in vitro in terms of progesterone production, though this

brought about by LH receptor down regulation.

The desensitized corpora lutea also showed marked decrease in mitochondrial cholesterol. Only part of the mitochondrial cholesterol is available to the side chain cleavage activity (see Dorrington, 1977). Hence the effect of a decrease in the cholesterol level of mitochondria on side chain cleavage activity is probably enhanced further. There was a significant decrease in pregnenolone production by mitochondria from desensitized corpora lutea. This defect in pregnenolone biosynthesis could be ameliorated by addition of cholesterol to the mitochondrial preparation: pregnenolone production by mitochondria from desensitized corpora lutea in the presence of exogenous cholesterol was nearly equal to that by control mitochondria. These results suggest that the cholesterol side chain cleavage activity is probably functionally intact, and the decrease in progesterone biosynthesis could be due to the decrease in the cholesterol pool in mitochondria which acts as the substrate for cholesterol side chain cleavage enzymes. Replenishment of the mitochondrial cholesterol pool may take a much longer time than that of tissue cholesterol. Levels of tissue cholesterol had returned to nearly control levels 24 h following desensitization treatment, but cholesterol in the mitochondria remained markedly reduced. There was no change in 3β-hydroxy-steroid dehydrogenase in desensitized corpora lutea but a significant increase in cAMP phosphodiesterase activity was noted (data not shown). This defect can cause further decrease in progesterone biosynthesis.

Thus down regulation of luteal tissue following administration of hCG occurs at at least two different levels—(i) loss of receptors which is perhaps mediated by prostaglandins and (ii) decrease in the cholesterol pool resulting in decrease in progesterone production.

Acknowledgements

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Mycobacterium leprae mediated stimulation of macrophages from leprosy patients and hydrogen peroxide production

JOLLY MAROLIA and P. R. MAHADEVAN

The Foundation for Medical Research, 84-A, R. G. Thadani Marg, Worli, Bombay 400 018, India

Abstract. Macrophages cultured from the peripheral blood of normal individuals, tuberculoid leprosy patients and long-term-treated, bacteriologically negative lepromatous leprosy patients are able to release hydrogen peroxide on stimulation with *Mycobacterium leprae*. Macrophages from lepromatous leprosy patients who are bacteriologically positive produce considerably lower levels of hydrogen peroxide, even though stimulation of these cells with *Mycobacterium leprae* is definitely demonstrable. This differential stimulation of macrophages appears to be largely specific to *Mycobacterium leprae*. There is also a good indication that decreased stimulation of macrophages from positive patients could be due to an after-effect of infection. It is possible that while other factors aid survival of *Mycobacterium leprae* in the macrophages, hydrogen peroxide may not be as effective in the killing of the bacteria in infected patients as it would be, perhaps, in other infections.

Keywords. Hydrogen peroxide; normal persons; leprosy patients; deficiency; role in production.

Introduction

Macrophages are credited with the ability to inactivate and kill bacteria that have been phagocytosed. The ability of macrophages in this regard is a consequence of their activation through immune processes mediated by T-lymphocytes and their products (Mackaness, 1969). Such activation of macrophages results in the release of reactive chemical species such as hydrogen peroxide (H_2O_2), superoxide (O_2^-) anions and hydroxyl radicals (OH·). This has been explained as the principal *in vivo* process of killing intracellular pathogens (Jackett *et al.*, 1978; Klebanoff, 1982; Nathan *et al.*, 1979; Walker and Lowrie, 1981).

Mycobacterium leprae, the causative organism in leprosy, has been shown to be susceptible to hydrogen peroxide in the presence of myeloperoxidase' and halides (Klebanoff and Shepard, 1984), and direct treatment and incubation with 0·08% of H_2O_2 (Sharp *et al.*, 1985). Even though both these reports show susceptibility of *M. leprae* to H_2O_2, they are under two different conditions. Recently, Sharp and Banerjee (1985) reported that hydrogen peroxide is produced by monocytes from all types of leprosy patients, and suggested further that macrophages from leprosy patients are competent to inactivate *M. leprae*. According to them, a T-lymphocyte defect probably contributes to the susceptibility of individuals to *M. leprae*.

This paper reports studies on the effect of addition of *M. leprae* to well-matured macrophages from various types of leprosy patients and normal healthy persons on

by macrophages.

We recently reported that production of O_2^- is defective in macrophages types of leprosy patients on contact with live *M. leprae* in contrast to what observed with macrophages from normal healthy individuals (Marolia Mahadevan, 1987). Some recent observations have also helped us to elucidat role of H_2O_2, O_2^- and OH^- in the inactivation of *M. leprae* inside the phagocy both normal and leprosy patients (Jolly Marolia and P. R. Mahadevan, unpubl results).

Materials and methods

Patients

Leprosy patients attending some of the clinics in Bombay, specially Acv Leprosy Hospital, donated blood voluntarily for our studies. They were according to the classification of Ridley and Jopling (1966). The lepromatous were primarily BL and some, LL; the tuberculoid type were primarily BT and TT. Among the lepromatous type we studied two groups: (i) long-term-treated years treatment) bacteriologically negative (smear-negative, $B(-)LL$) patient (ii) short-term-treated or untreated bacteriologically positive (smear-po $B(+)LL$) patients. In the tuberculoid leprosy group both untreated and tr patients were studied and treated as a single category. The normal controls those healthy individuals in Bombay who had various degrees of exposu *M. leprae* from the environment. These healthy controls were neither close cor nor people who had regular contacts with leprosy patients. A minimum individuals have been studied in each category, and these are considered different experiments.

M. leprae bacilli were obtained from infected tissues of armadillo (suppli Dr E. Storrs, Florida, USA). Bacteria were removed from infected tissue by rep rinsing of the tissue in sterile saline (1 N). After centrifugation of the rinsing liq 4500 g for 15 min, a significant number of acid-fast-staining bacteria was obt Such bacilli have been found to be largely free from tissue contaminants by r scopic analysis. Further, incubation of such bacilli with concentrated per solution released no visible bubbles of H_2O_2. Lastly, such bacteria, as the data indicate, did not show differences in macrophage-stimulating ability when use or as heat-killed cells. If host catalase was present as a contaminant, one expect a lower stimulation (H_2O_2 measured) with live *M. leprae* than with an number of heat-killed *M. leprae*, because in a heat-killed preparation, the en would have been inactivated. The presence of viable bacteria was confirmed in preparation before use by using fluorescein diacetate according to the meth Kvach *et al.* (1984).

M. leprae cells were heat-killed by autoclaving at 121°C and 15 lbs/sq in 30 min.

Preparation of macrophage cultures

n (25 units/ml) and 6% dextran. The blood was allowed to settle at 37°C for
.. Plasma and buffy coat were transferred to a sterile tube and centrifuged at
or 5 min. The sedimented pellet of leucocytes was washed once with minimum
al medium (MEM) (Gibco, UK) and the cells were then suspended in MEM
mented with human AB-type serum (added to 40% concentration). Aliquots of
f the suspension were transferred to 35 mm sterile Falcon Petri dishes. In
l experiments it was observed that 5 ml of the cell suspension contained, on an
e, $0.8–1 \times 10^6$ macrophages. After 24 h of incubation at 37°C in 5% CO_2
phere, non-adherent cells were removed by draining the liquid. The culture
m was changed every 48 h thereafter and the culture was maintained for 5
This resulted in a fairly uniform layer of adherent, esterase-positive, phagocytic
phages.

for H_2O_2

nature macrophage cultures were washed thrice with Eagle's balanced salt
n (EBSS). To the cultures were added phenol red (0.2 mg/ml, 1 ml), horseradish
dase (Sigma Chemical Co., USA) (2 units/ml) and M. leprae live or autoclaved
0^6/dish). Since we had earlier determined that the culture contained $0.8 –$
0^6 macrophages, bacteria and macrophages were in a ratio of about 50:1. All
ns were prepared in EBSS. The total volume of the incubation mixture was
To control Petri dishes 20 μl of 1 N NaOH was added before the incubation in
to kill the cells and block H_2O_2 production. The cultures were incubated at
or 3 h after the addition of M. leprae and the reaction was stopped by adding
f 1 N NaOH. The colour developed at the end of the reaction was measured as
pance at 610 nm and H_2O_2 was quantitated. The number of macrophages in
ulture dish was counted microscopically after scraping and resuspending them.
ssay used here is essentially the same as the one described by Pick and Mizel
. The level of H_2O_2 is expressed as nmol/h/10^6 macrophages. This was
nined from the extinction coefficient calculated from a standard curve. The
of values from 5 different experiments and the standard deviation of the mean
calculated and the statistical significance of difference between values for
nt samples was determined by the Student's 't' test.

specificity of the low response to M. leprae in B(+)LL macrophages
red to B(−)LL macrophages was tested using several other mycobacteria in
of M. leprae (listed in table 5). The assay system was similar to the one
bed for M. leprae.

effect of already phagocytosed live M. leprae on H_2O_2 production by the
phages carrying the bacilli upon addition of heat-killed M. leprae was also
nined. This was done by stimulating B(−)LL macrophages with heat-killed
rae after they had phagocytosed live or heat-killed M. leprae for various
ls.

on exposure to *M. leprae*.

	Tuberculoid leprosy			Normal		
	Control	Macrophage + heat-killed *M. leprae*	Macrophage + live *M. leprae*	Control	Macrophage + heat-killed *M. leprae*	Macrophage + live *M. leprae*
	(A)	(B)	(C)	(D)	(E)	(F)
	10·7	50·7	60·0	16·0	66·7	113·3
	10·7	29·3	46·7	24·0	57·3	106·6
	16·0	48·0	72·0	10·7	80·0	124·0
	21·3	53·3	66·7	16·0	72·0	116·0
	10·7	48·0	69·3	13·3	76·0	96·0
Mean ± SD	13·88 ± 4·7	45·9 ± 9·5	62·9 ± 10·1	16 ± 5	70·4 ± 8·8	111·18 ± 10·5
M. leprae preparation with tissue contamination (catalase)						
	12·0	50·0	8·0*			

*Average values of results of 4 separate experiments, nmol/h/10^6 macrophages.

Significance of difference: A–C, $P<0.001$; D–F, $P<0.001$; A–B, $P<0.001$; D–E, $P<0.001$; A–D, not significant.

tuberculoid leprosy patients. The data show that there is very good stimulation by *M. leprae*, as indicated by the increase in the level of H_2O_2. However, H_2O_2 production by macrophages from tuberculoid leprosy patients was considerably less than that by macrophages from healthy individuals with both live *M. leprae* (62·9 nmol compared with 111·18 nmol) and heat-killed *M. leprae* (45·9 nmol compared with 70·4 nmol). Further, killed *M. leprae* caused less stimulation than live *M. leprae* in both types of macrophages. The killed *M. leprae* preparation was used to check for contaminating catalase as well as to determine the ability of such *M. leprae* to stimulate the macrophages. The results clearly indicate that there was no contaminating catalase in the live *M. leprae* preparation because if catalase was present, the H_2O_2 measured would be less than in the control. The effect of contaminating catalase is clearly seen in the H_2O_2 levels when a contaminated preparation is used (table 1).

Data from similar experiments with macrophages from the two types of lepromatous leprosy patients are presented in table 2. From a comparison of data in tables 1 and 2, it is clear that macrophages from bacteriologically positive lepromatous leprosy patients are stimulated much less by *M. leprae* compared to macrophages from normal individuals or tuberculoid leprosy patients ($P<0.001$). On the other hand, macrophages from long-term-treated bacteriologically negative patients were capable of being stimulated very well by both live and heat-killed *M. leprae* (89 and 83 nmol H_2O_2 respectively). This result is thus similar to what was observed with macrophages from normal individuals or tuberculoid leprosy patients. However, the B(−)LL macrophages differ from the normal and tuberculoid leprosy in another aspect: the former were stimulated to similar extents by live and heat-killed bacilli whereas the latter types were stimulated to a greater extent by live bacilli. The B(+)LL macrophages produced low levels of H_2O_2 even though phagocytosis by

Table 2. H$_2$O$_2$ release by macrophages from B(+)LL and B(-)LL lepromatous leprosy patients on exposure to *M. leprae*.

	B(+)LL			B(-)LL		
	Control	Macrophage + heat-killed *M. leprae*	Macrophage + live *M. leprae*	Control	Macrophage + heat-killed *M. leprae*	Macrophage + live *M. leprae*
	(A)	(B)	(C)	(D)	(E)	(F)
	10·7	16·0	23·9	16·0	88·0	93·3
	10·7	13·3	23·9	13·3	80·0	88·0
	10·7	23·9	26·7	16·0	73·3	86·6
	12·0	18·7	23·9	16·0	89·3	88·0
	13·3	23·9	26·7	13·3	85·3	89·3
Mean ± SD	11·48 ± 1·16	19·16 ± 4·73	25·02 ± 1·53	14·9 ± 1·98	83·18 ± 6·6	89·04 ± 2·6
M. leprae preparation with tissue contamination (catalase)						
	12·0	20·0	5·0	15·0	75·0	07·0*

*Average values of results of 4 separate experiments, nmol/h/10^6 macrophages.
Significance of difference: A–B, $P > 0.02$ (not significant); A–C, $P < 0.001$ (significant); D–E, $P < 0.001$; D–F, $P < 0.001$; A–D, not significant.

Table 3. Phagocytic indices for macrophages at various times after infection*.

Macrophages from	Phagocytic index (h)				
	1	3	5	7	24
Normal	8 ± 1·01	16 ± 2·90	96 ± 04·3	150 ± 10·60	720 ± 27·50
Tuberculoid lerrosy	8 ± 1·02	20 ± 2·99	100 ± 05·0	140 ± 10·00	900 ± 28·70
B(+)LL	6 ± 1·00	40 ± 5·00	95 ± 03·5	100 ± 05·00	840 ± 24·49
B(-)LL	20 ± 3·00	60 ± 3·50	200 ± 10·7	240 ± 10·19	1800 ± 86·02

*5×10^6 *M. leprae* per Leighton tube culture of macrophages.

$$\text{Phagocytic index} = \frac{\text{Average number of bacilli per macrophage}}{\text{Total number of macrophages with phagocytosed } M. \text{ leprae}}$$

Values are means of results of 5 experiments ± SD. P (B(-)LL–Normal) < 0.005.

To confirm that the levels of released H$_2$O$_2$ recorded were realistic, some control experiments were also carried out. The levels of H$_2$O$_2$ released by macrophages from leprosy patients after stimulation by phorbol myristate acetate (PMA) were determined. It is clear from the data in table 4 that PMA stimulated the macrophages of B(-)LL and tuberculoid leprosy patients (55 and 58 nmol) and that this stimulation is blocked in the presence of either added catalase or contaminating catalase.

Four other species of mycobacteria could not cause differential stimulation of macrophages from B(-)LL and B(+)LL patients (table 5). *M. vaccae* showed a tendency to discriminate between these macrophages. In all these cases phagocytosis of the bacteria was quite satisfactory and comparable to each other (data not shown).

The ability of B(-)LL macrophages to be stimulated by heat-killed *M. leprae* is

agents.

Stimulating agent	Macrophages from	
	B(−)LL	Tuberculoid leprosy
None (control)	13·55	13·90
PMA (1 μg/ml)	55·00	58·20
PMA (1 μg/ml) + catalase (100 μg/ml)	00·00	13·30
Heat-killed M. leprae	56·00	48·50
Heat-killed M. leprae + catalase (100 μg/ml)	15·90	00·00
Live M. leprae (with tissue contamination)	00·00	26·50
Live M. leprae (normal exptl. sample)	00·00	58·23

Values are averages of results of 3 experiments, nmol/h/10⁶ macrophages.

Table 5. H_2O_2 release by macrophages from leprosy patients on stimulation by various species of mycobacteria.

Stimulating bacteria	Macrophages from		
	B(+)LL	B(−)LL	Tuberculoid leprosy
None	13·30 ± 2·4	15·2 ± 01·3	12·60 ± 02·8
Heat-killed M. leprae	23·90 ± 0·0	72·8 ± 17·2	38·50 ± 13·3
Live M. leprae	26·50 ± 0·0	80·0 ± 18·8	56·60 ± 14·0
M. avium	19·41 ± 0·6	26·0 ± 06·8	18·80 ± 11·2
M. intracellulare	18·80 ± 2·8	29·1 ± 08·3	17·14 ± 05·9
M. scrofulaceum	18·40 ± 1·3	29·0 ± 06·1	16·80 ± 06·6
M. vaccae	22·30 ± 1·1	62·8 ± 02·1	32·80 ± 01·3
M. bovis (BCG)	22·60 ± 5·5	38·2 + 04·1	22·70 ± 05·5

Values are means of results of 3 experiments ± SD nmol/h/10⁶ macrophages.

Table 6. H_2O_2 release by B(−)LL macrophages carrying phagocytosed live or heat-killed M. leprae on stimulation with heat-killed M. leprae.

Treatment	1	2	3	4	Mean ± SD
None (control)	12·9	13·3	13·3	13·3	13·3 ± 00·2
Heat-killed M. leprae (A)	90·6	90·0	84·8	79·6	86·4 ± 05·3
Live M. leprae (24 h) + heat-killed M. leprae (3 h) (B)	69·2	—	36·9	—	53·1 ± 22·0
Live M. leprae (48 h) + heat-killed M. leprae (3 h) (C)	64·1	74·4	68·6	—	69·0 ± 05·2
Live M. leprae (96 h) + heat-killed M. leprae (3 h) (D)	—	—	—	53·1	53·1
Heat-killed M. leprae (24 h)	80·0	—	77·3	—	78·6 + 01·9

phagocytosed any bacilli (table 6, compare means of rows A and E). The presence of phagocytosed *M. leprae* could be shown by staining the intracellular bacteria.

Discussion

The striking observation is that macrophages from healthy controls, tuberculoid leprosy patients and long-term-treated, bacteriologically free, 'cured' lepromatous leprosy patients have the ability to produce H_2O_2 on encountering *M. leprae* in *in vitro* culture. In contrast, macrophages from bacteriologically positive lepromatous leprosy patients show poor ability to respond to *M. leprae* by producing H_2O_2. It appears that the poor response to *M. leprae* in these macrophages is an after-effect of infection. The other interesting feature is that macrophages from tuberculoid leprosy patients are comparatively less responsive than macrophages from healthy individuals or 'cured' lepromatous leprosy patients. We are unable to identify the reason for this.

In macrophages from normal individuals, live *M. leprae* induced 60% more H_2O_2 than heat-killed bacilli whereas in tuberculoid patients live *M. leprae* induced only 37% more H_2O_2 than heat-killed bacilli. The greter stimulating ability of live bacilli could be interpreted as a result of the conversion of induced O_2^- to H_2O_2 by superoxide dismutase (SOD) present in live *M. leprae*. However, in $B(-)LL$ patients, live *M. leprae* induce levels of H_2O_2 that are similar to those induced by heat-killed *M. leprae*. Since SOD of *M. leprae* can convert O_2^- to H_2O_2, high levels of H_2O_2 indicate high levels of O_2^- production in macrophages from normal individuals; correspondingly, there may be much less O_2^- in macrophages from tuberculoid leprosy patients and possibly very little or nil in macrophages from $B(-)LL$ patients when stimulated with live *M. leprae*. Recent observations by us have shown that more O_2^- is produced by macrophages from normal individuals on encountering live *M. leprae* than by the other types of macrophages (Marolia and Mahadevan, 1987).

The levels measured by us appear to be realistic and comparable to other reported values (Nathan and Root 1977; Nathan *et al.*, 1983; Kaplan *et al.*, 1980). Stimulation with PMA also resulted in similar levels of H_2O_2 and added catalase or contaminating tissue catalase reduced the H_2O_2 to very low levels, indicating a true production of H_2O_2. This was the case with macrophages from both $B(-)LL$ and tuberculoid leprosy patients. In several samples studied by Sharp and Banerjee (1985) in all categories of patients, there was no stimulation at all. We have used 5-day-matured macrophages and 3 h exposure to *M. leprae*. Our results clearly point out the inability of macrophages of infected patients to produce as much hydrogen peroxide as macrophages from the other types of individuals. Most of the studies reported in the literature, except the recent report of Kaplan *et al.* (1986), have not distinguished LL patients as we have done. In the study of Kaplan *et al.* (1986), one patient had zero bacterial load and the macrophages from this patient showed higher stimulation than macrophages from patients with $3+$ or $4+$ bacteriological load.

The phagocytic abilities of macrophages from healthy individuals and those from $B(+)LL$ patients are similar to each other. The ratio of *M. leprae* cells to macrophages in the cultures was approximately $50:1$ in all the experiments. Thus the

live *M. leprae* for 24–96 h results in the macrophages becoming less responsive to heat-killed *M. leprae*. This is indicative of some changes induced by the phagocytosed live *M. leprae* in the B(−)LL macrophages. The 20–40% reduction in the response of such macrophages to heat-killed *M. leprae* could be due to these changes. A possible reason for the poor H_2O_2 production *in vivo* in macrophages of B(+)LL patients is indicated by this *in vitro* experiment.

It appears that the low level of stimulation of B(+)LL macrophages is largely specific for *M. leprae*. Other mycobacteria are not able to distinguish between macrophages from the different groups of patients, although *M. vaccae* showed some discrimination between B(+)LL and B(−)LL macrophages.

It is possible that H_2O_2 by itself may not be playing significant role in inactivating *M. leprae* inside macrophages in leprosy because of two observations:

(i) In long-term-treated patients where H_2O_2 production is high, *M. leprae* are phagocytosed and remain metabolically active.
(ii) Changes caused by phagocytosed live *M. leprae* eventually reduce the ability of macrophages to be stimulated by fresh *M. leprae* to produce sufficient H_2O_2 even if it were to kill or inactivate the bacteria.

Such events may take place during *in vivo* infection also. Thus O_2^- and concomitant immune stimulation may be the basic requirements. Recent data (Marolia and Mahadevan, 1987) point to the importance of O_2^- as a critical component of reactive oxygen species involved in bacterial killing. O_2^- was not produced in all leprosy patients on encountering live *M. leprae*. Additional information regarding the role of hydroxyl radicals and the loss of viability of *M. leprae* in macrophages has also been obtained. This has clearly indicated that O_2^- as well as OH· radicals are much more important than H_2O_2 in killing *M. leprae* inside the macrophages (Jolly Marolia and P. R. Mahadevan, unpublished results).

Acknowledgements

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Assay of superoxide dismutase activity in animal tissues

ANURADHA NANDI and I. B. CHATTERJEE*

Department of Biochemistry, University College of Science, 35, Ballygunge Circular Road, Calcutta 700 019, India

Abstract. Convenient assays for superoxide dismutase have necessarily been of the indirect type. It was observed that among the different methods used for the assay of superoxide dismutase in rat liver homogenate, namely the xanthine–xanthine oxidase ferricytochrome c, xanthine–xanthine oxidase nitroblue tetrazolium, and pyrogallol autoxidation methods, a modified pyrogallol autoxidation method appeared to be simple, rapid and reproducible. The xanthine–xanthine oxidase ferricytochrome c method was applicable only to dialysed crude tissue homogenates. The xanthine–xanthine oxidase nitroblue tetrazolium method, either with sodium carbonate solution, pH 10·2, or potassium phosphate buffer, pH 7·8, was not applicable to rat liver homogenate even after extensive dialysis. Using the modified pyrogallol autoxidation method, data have been obtained for superoxide dismutase activity in different tissues of rat. The effect of age, including neonatal and postnatal development on the activity, as well as activity in normal and cancerous human tissues were also studied. The pyrogallol method has also been used for the assay of iron-containing superoxide dismutase in *Escherichia coli* and for the identification of superoxide dismutase on polyacrylamide gels after electrophoresis.

Keywords. Superoxide dismutase; animal tissues; erythrocyte; human cancer tissues.

Introduction

Toxicity by oxygen radicals has been suggested as a major cause of cancer, aging, heart disease and cellular injury in hepatic and extrahepatic organs (Hartman, 1981; Troll and Weisner, 1985; Gram *et al.*, 1986). There is compelling evidence that superoxide dismutases (SOD, EC 1·5·1·1) are essential for biological defence against the superoxide anion (Fridovich, 1983). A variety of methods have been developed to assay SOD (Flohe and Ötting, 1984), but most of these are based on pure SOD samples. Practically, many of these methods are not suitable for the assay of SOD in animal tissues, which often contain materials that interfere with SOD assay. As a result, authentic data on SOD activities in different animal tissues are not available. Further, published reports regarding the effect of aging on tissue SOD activities are controversial (Leibovitz and Siegel, 1980; Steinhagen-Thiessen *et al.*, 1986). In microorganisms, SOD activity increased markedly with elevation of oxygen concentration (Fridovich, 1974). It would, therefore, be interesting to determine SOD activity in animal tissue at birth and during postnatal development, *i.e.*, when the animals are exposed to atmospheric oxygen. Since there is evidence that oxygen radicals play a role in tumour promotion (Troll and Weisner, 1985), it would also be interesting to determine the SOD status in human cancer. In this communication we have given

method, data have been obtained on SOD activities in different tissues of rats, the effect of age on SOD activities in rat tissues, and SOD activities in normal and cancerous human tissues.

Convenient assays for SOD have necessarily been of the indirect type (Flohe and Ötting, 1984; Beyer and Fridovich, 1987). Among these, the xanthine–xanthine oxidase ferricytochrome c (X/XOD/Cyt c^{3+}) method described by McCord and Fridovich (1969) is the first and the most widely used method. However, for the assay of SOD in animal tissues, the X/XOD/Cyt c^{3+} method should be used with caution because (i) ascorbic acid present in animal tissues (approximately 10^{-3} M) is a scavenger of superoxide (O_2^-) (Nishikimi, 1975; Nandi and Chatterjee, 1987) and also reduces cytochrome c chemically and interferes with the SOD assay, and (ii) cytochrome c oxidase activity present in the tissues may be mistaken for SOD activity, since reoxidation of reduced cytochrome c mimics an inhibition of cytochrome c reduction. Also, uric acid, the product of the action of xanthine oxidase on xanthine, is a scavenger of oxygen free radicals (Ames et al., 1981). Ascorbic acid may be removed by extensive dialysis. However, when the number of samples is large or when the sample size is small (as in human tissues), dialysis is not suitable for routine purposes. Interference of cytochrome c oxidase may be eliminated by using 10 μM potassium cyanide (KCN) in the assay mixture. But in practice, 10 μM KCN often does not fully inhibit cytochrome oxidase in crude tissue homogenates (Crapo et al., 1978). Moreover, Rigo et al. (1975) have shown that cyanide, even at a concentration of 1.77×10^{-6} M, may affect SOD. Higher concentrations of KCN significantly inhibit Cu,Zn-SOD. Azzi et al. (1975) recommended the use of acetylated ferricytochrome c in place of ferricytochrome c, because acetylated ferricytochrome c is not recognized as a substrate by cytochrome c oxidase. But this is not suitable for routine purposes. Preparation of acetylated ferricytochrome c is time-consuming, and the yield and stability of the acetylated product are low. Further, the rate of change of absorbance using acetylated ferricytochrome c is markedly low: we observed that the change of absorbance of 0.02 per min at 550 nm using X/XOD/Cyt c^{3+} became 0.007 when ferricytochrome c was replaced by acetylated ferricytochrome c.

Besides ferricytochrome c, nitroblue tetrazolium (NBT) is also used as a detector of O_2^- generated by the X/XOD system (Beauchamp and Fridovich, 1971). However, the X/XOD/NBT assay is better suited for monitoring SOD in polyacrylamide gels (Beauchamp and Fridovich, 1971). We have observed that the spectrophotometric assay of SOD using the X/XOD/NBT method is not applicable to crude tissue homogenates. As will be seen in this paper, NBT is chemically reduced by low concentrations of ascorbic acid present in tissue homogenates. This is particularly important for (i) the adrenal gland where ascorbic acid concentration is comparatively high, and (ii) lung and brain tissues where SOD activities are comparatively low and relatively high amounts of tissue homogenates are to be used in the assay mixture. Moreover, data presented in this communication indicate that even extensively dialysed tissue homogenate produces non-specific interference in the X/XOD/NBT assay of SOD. Misra and Fridovich (1972) reported an assay for SOD based on the ability of SOD to inhibit the autoxidation of epinephrine at

have observed that in a system containing 3×10^{-4} M epinephrine and 0·05 M sodium carbonate solution, pH 10·2, 1×10^{-5} M ascorbic acid or 5×10^{-5} M GSH completely inhibits the autoxidation of epinephrine.

We have observed that reliable and reproducible results are obtained in the assay of SOD in crude tissue homogenates by the pyrogallol autoxidation method of Marklund and Marklund (1974) only after making some minor but significant modifications such as the use of pH 8·5 instead of pH 8·2 in the assay mixture and allowance of a lag period of one and a half min to allow the steady state of oxidation of pyrogallol to be attained before taking the initial reading. The method is simple and is not affected by the concentrations of ascorbic acid and glutathione present in tissue homogenates. The pyrogallol autoxidation method can also be used for the assay of Fe-SOD in *Escherichia coli*. We have further observed that the pyrogallol method can be used to detect SOD on polyacrylamide gels following electrophoresis.

Materials and methods

Xanthine and xanthine oxidase (grade I), cytochrome c, SOD, NBT, Triton X-100 and Tris were purchased from Sigma Chemical Co., St Louis, Missouri, USA. Catalase was obtained from CSIR Centre for Biochemicals, New Delhi, and was found to be free from SOD contamination. Ascorbic acid was a product of Sarabhai M. Chemicals, Bombay. Sodium carbonate, A.R., and hydrogen peroxide (H_2O_2) were obtained from Glaxo Laboratories, Bombay. Pyrogallol, a product of E. Merck, Germany, was purified by sublimation. Cacodylic acid was purchased from Fisher Scientific Company, Fair Lawn, New Jersey, USA; di-ethylenetriaminepentaacetic acid (DTPA), A.R., from Koch-Light Laboratories Ltd, England; KCN from Burgoyne Urbidges, India, and sodium dodecyl sulphate (SDS) from British Drug House, Bombay.

For SOD assay, the tissue homogenate (1:9 for lung and adrenal gland, w/v, and 1:4 for other tissues) was prepared in ice-cold 0·25 M sucrose solution containing 0·5% Triton X-100. The crude homogenate was centrifuged at 34,880 g for 30 min and the supernatant was used. Sucrose (0·25 M) and Triton X-100 (0·5%) did not interfere with the SOD assay mentioned in the text. The amount of tissue homogenate needed for SOD assay by the pyrogallol method was 2–5 μl in the case of liver, 5–10 μl for kidney, and 25–40 μl for lung. Higher amounts of tissue homogenates were needed for assays in tissue from newly born or developing rats. When SOD was assayed by the X/XOD/Cyt c^{3+} and X/XOD/NBT methods, the 34,880 g supernatant was dialysed against 20 volumes of 10 mM potassium phosphate buffer, pH 7·2, for 8 h with 8 changes of buffer at 4°C.

SOD assay in erythrocytes was done in Tsuchihasi extract of erythrocyte haemolysate (Crapo et al., 1978). The red cells were haemolysed with 3 volumes of cold glass-distilled water. The amount of haemoglobin (Hb) present in the erythrocyte haemolysate was estimated by Drabkin's method (Richterich, 1969). Ninety-five to 98% of pure SOD added to erythrocyte haemolysate could be

Assay of SOD by X/XOD/Cyt-c method was carried out according to McCord and Fridovich (1969) and Crapo *et al.* (1978). SOD assay by X/XOD/NBT method was carried out following the method of Beauchamp and Fridovich (1971) as well as that of Oberly and Spitz (1984). SOD assay by the pyrogallol autoxidation method was carried out following the procedure of Marklund and Marklund (1974). The assay system contained 1 mM DTPA, 40 μg catalase, 50 mM air-equilibrated Tris cacodylate buffer, pH 8·5 and tissue homogenate or Tsuchihasi extract of erythrocyte in a final volume of 2 ml. The reaction was initiated by the addition of 100 μl of freshly prepared 2·6 mM pyrogallol solution in 10 mM HCl to attain a final concentration of pyrogallol of 0·13 mM in the assay mixture. The assay mixture was transferred to a 1·5 ml cuvette and the rate of increase in the absorbance at 420 nm was recorded for 2 min from 1 min 30 s to 3 min 30 s in a Hitachi Model 200–20 double beam spectrophotometer with recorder. The lag of 1 min 30 s was allowed for steady state of autoxidation of pyrogallol to be attained. The allowance of this lag period was very important for reproducibility of results. The concentration of pyrogallol was so adjusted that the rate of change of absorbance per min was approximately 0·020–0·023. The increase in the absorbance at 420 nm after addition of pyrogallol was inhibited by the presence of SOD. One unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol autoxidation per 3 ml of assay mixture. Results have been expressed in units per g tissue or per mg protein for tissue homogenate and units per g Hb for erythrocyte haemolysate. All the experiments were carried out in an air-conditioned room at 25°C. Protein was estimated according to the method of Lowry *et al.* (1951).

To determine the amounts of Cu, Zn-SOD and Mn-SOD in tissues, 2 mM KCN solution was added to the assay mixture to inhibit Cu, Zn-SOD; Mn-SOD remains unaffected (Fridovich, 1974). However, in lung tissue SDS was used instead of KCN, because KCN gave an erroneously high value for Mn-SOD when the tissue was contaminated with Hb. We observed that in the presence of 2 mM KCN, 2·5 μg or more Hb, when added to the 2 ml assay mixture, mimicked about one unit of Mn-SOD in the pyrogallol method. For the determination of Mn-SOD in lung tissues, the homogenate was pre-incubated with 2% SDS at 37°C for 30 min before addition to the assay mixture. SDS inhibits Mn-SOD; Cu, Zn-SOD remains unaffected (Geller and Winge, 1984). SDS (2%) did not interfere with the autoxidation of pyrogallol. The decrease in the total activity after addition of SDS represents Mn-SOD. The Mn-SOD and Cu, Zn-SOD were thus calculated as the difference between the total SOD activity and the activity due to Cu, Zn-SOD and Mn-SOD, respectively. It was found that 2 mM KCN inhibited the autoxidation of pyrogallol by about 15%. So, for the determination of Mn-SOD in tissues other than lung, one control experiment was carried out by recording the increase of absorbance of autoxidation of pyrogallol at 420 nm in the presence of 2 mM KCN. The 50% inhibition after addition of tissue homogenate was calculated on this basis. The pyrogallol method was also used for the assay of Fe-SOD in *E. coli* extract by adding 0·5 mM H_2O_2 to the assay mixture. H_2O_2 inactivated Fe-SOD, but not Mn-SOD (Bridges and Salin, 1981). The excess of H_2O_2 was decomposed by addition of excess catalase. To the assay mixture containing 1 mM DTPA and

5 min, 0·3 ml of catalase solution (120 μg) was added and the solution was left for another 10 min, with occasional shaking. Then 100 μl of 2·6 mM pyrogallol was added and the rate of increase in the absorbance at 420 nm per min was recorded as before. A control experiment was run side by side in the absence of *E. coli* extract.

Polyacrylamide disc gel electrophoresis of tissue homogenate or Tsuchihasi extract of erythrocyte haemolysate was performed in Tris–glycine buffer, pH 8·3, according to the method of Davis (1964). After electrophoresis, each gel was soaked in a tube containing a mixture of 3 ml of 50 mM potassium phosphate buffer, pH 6·5, and 2 ml of a solution of 2 mg NBT in water for 1 h in the dark. Then the gels were transferred into separate staining tubes each containing a mixture of 5 ml of 50 mM potassium phosphate buffer, pH 6·5, and 0·4 ml of 0·25 mM pyrogallol solution. The gels were stained violet with achromatic zones in the region of SOD.

Results and discussion

SOD assay by the pyrogallol method is dependent on the pH of the assay mixture. The rate of increase in absorbance at 420 nm per min increased with increasing pH from 8·2–8·9. We observed that better sensitivity and reproducibility were obtained when the pH of the assay mixture was kept at 8·5. At this pH, the autoxidation of pyrogallol was inhibited about 88% by 2 pyrogallol units of bovine erythrocyte SOD indicating that the reduction was mediated *via* O_2^-. When applied to liver homogenate, the absorbance at 420 nm was linear with time up to 3 min.

Figure 1 shows that when per cent inhibition is plotted against amount (μl) of liver homogenate, the graph is linear from about 28–70% inhibition, 50% inhibition being obtained with 5 μl homogenate. When the liver homogenate was replaced by pure bovine erythrocyte SOD diluted to identical activity, *i.e.*, 50% inhibition per 5 μl SOD solution, an overlapping plot was obtained. An overlapping plot was also obtained with a fifty–fifty (v/v) mixture of liver homogenate and pure SOD solution. When the SOD assay was carried out by the X/XOD/Cyt c^{3+} method, a linear graph was obtained from about 20–63% inhibition. In this case again, pure SOD solution or a fifty–fifty mixture (v/v) of liver homogenate and SOD solution gave an overlapping graph. However, when 50% inhibition was taken as one unit of SOD, the unit obtained by the pyrogallol method was approximately two times the value obtained by the X/XOD/Cyt c^{3+} method. This has also been mentioned by Beyer and Fridovich (1987). SOD assay by the X/XOD/NBT method cannot be carried out in undialysed tissue homogenate, because ascorbic acid in the homogenate interferes with this method. The average ascorbic acid contents of rat adrenal gland, brain and lung were 1600, 275 and 200 μg per g tissue respectively. If undialysed tissue homogenates were added to the assay mixture, the average concentrations of ascorbic acid would have been 3·40 μM in the case of adrenal gland, 2·56 μM for brain, and 2·27 μM for lung. We observed that 2·5 μM ascorbic acid in the assay mixture containing sodium carbonate solution, pH 10·2, reduced NBT resulting in an increase of absorbance of 0·009 at 550 nm. The absorbance increased linearly with increasing concentration of ascorbic acid and this was not inhibited by SOD.
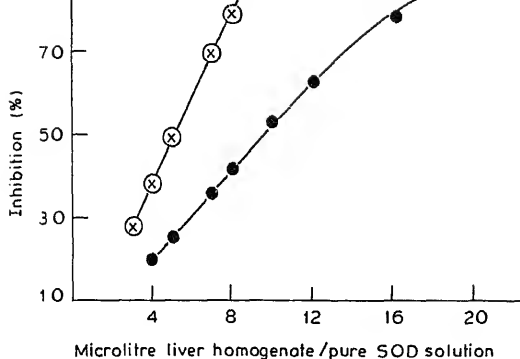
Figure 1. Per cent inhibition as a function of amount (μl) of liver homogenate and pure SOD solution. ($\times$) and ($\bigcirc$) represent liver homogenate solution and pure SOD solution, respectively, assayed by the pyrogallol method. ($\bullet$) represents liver homogenate or pure SOD solution assayed by the X/XOD/Cyt c^{3+} method. Details of the pyrogallol method are given under materials and methods. The assay mixture for X/XOD/Cyt c^{3+} method contained, in a final volume of 2 ml, 50 mM potassium phosphate buffer pH 7·8, 0·1 mM EDTA, 0·1 mM ferricytochrome c, 0·5 mM xanthine and the amount of xanthine oxidase (about 2·5 mU) required to obtain a change of absorption of about 0·02 per min at 550 nm. The amount of xanthine oxidase needed varied with different lots of xanthine oxidase obtained from Sigma Chemical Co. The reaction was initiated by the addition of xanthine oxidase. The assay mixture was quickly transferred to a 1·5 ml cuvette and readings were taken against blank at 550 nm for 2 min from the 30th second after addition of xanthine oxidase. All the reagents were prepared in 50 mM potassium phosphate buffer, pH 7·8. The blank cuvette contained everything except xanthine oxidase.

components of the tissue. Contrary to the results obtained by the pyrogallol method or the X/XOD/Cyt c^{3+} method (figure 1), in the X/XOD/NBT method using sodium carbonate solution, pH 10·2, there was a decrease in per cent inhibition with increasing liver homogenate concentration (figure 2). While in the X/XOD/Cyt c^{3+} method 4 μl liver homogenate produced 20% inhibition and 16 μl produced 78% inhibition, in the X/XOD/NBT method using sodium carbonate solution, pH 10·2, 4 μl homogenate produced 46% inhibition and 20 μl produced 13% inhibition.

Figure 2 also shows that when sodium carbonate solution was replaced by potassium phosphate buffer, pH 7·8, the X/XOD/NBT method could be applied only to pure SOD solution but not to the liver homogenate. Like in the case of sodium carbonate solution, in the potassium phosphate buffer also there was a decrease in per cent inhibition with increasing liver homogenate concentration: 4 μl homogenate produced 34% inhibition and 20 μl produced 25% inhibition. When the liver homogenate was diluted 5-fold, 4 μl of the diluted homogenate produced 16% inhibition and 1 μl practically produced no inhibition. Oberly and Spitz (1984) stressed the use of catalase in the X/XOD/NBT assay of SOD. Figure 2 shows that the use of catalase in the assay mixture produced about 44% recovery of per cent inhibition but the pattern of the plot remained similar. These results would indicate
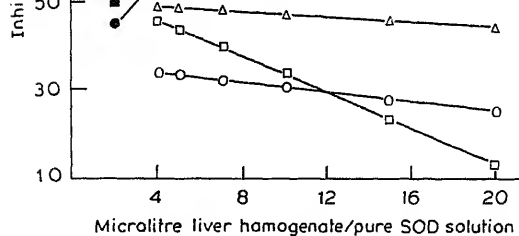
Figure 2. Per cent inhibition as a function of amount (μl) of liver homogenate and pure SOD solution. (■) and (●) represent pure SOD solution assayed by X/XOD/NBT method using sodium carbonate solution, pH 10·2, and potassium phosphate buffer, pH 7·8, respectively. (□) and (○) represent liver homogenate solution assayed by X/XOD/NBT method using sodium carbonate solution, pH 10·2, and potassium phosphate buffer, pH 7·8, respectively. ($\triangle$) represents liver homogenate solution assayed by X/XOD/NBT method using potassium phosphate buffer, pH 7·8, containing 40 μg catalase. The assay mixture for X/XOD/NBT method using sodium carbonate solution contained, in a final volume of 2 ml, 50 mM sodium carbonate solution pH 10·2, 1×10^{-4} M EDTA, $2\cdot5 \times 10^{-5}$ M NBT, and sufficient amount of xanthine oxidase (approximately 7 mU) to obtain a change of absorption of about 0·02 per min at 550 nm. The reaction was initiated by the addition of 1×10^{-4} M (final concentration) xanthine. The assay mixture for X/XOD/NBT method using potassium phosphate buffer contained, in a final volume of 2 ml, 50 mM potassium buffer pH 7·8, 1 mM DTPA, 1×10^{-4} M NBT and xanthine oxidase (approximately 1·5 mU) to obtain a change of absorption of about 0·02 per min at 550 nm. The reaction was initiated by the addition of 1×10^{-4} M (final concentration) xanthine. The assay mixture was quickly transferred to a 1·5 ml cuvette and readings were taken against blank at 550 nm for 2 min from the 30th second after addition of xanthine. The blank cuvette contained everything except xanthine.

potassium phosphate buffer, pH 7·8. It was further observed that in the X/XOD/NBT assay, irrespective of the buffer used, there was a decrease in per cent inhibition with increasing amount of liver homogenate added to pure SOD solution. In the absence of liver homogenate, 5 μl of pure SOD solution (equivalent to one pyrogallol unit of SOD) produced 65% inhibition with sodium carbonate solution, pH 10·2, and 60% inhibition with potassium phosphate buffer, pH 7·8. After addition of 12 μl liver homogenate, the per cent inhibition dropped to 35 and 33%, respectively. This might be due to some non-specific reduction of NBT by certain reductants including NBT reductase and a number of dehydrogenases usually present in crude tissue homogenates. Therefore, the data published previously (Peeters–Jorris *et al.*, 1975) on SOD activities in different tissues of rats assayed by the X/XOD/NBT method are questionable.

Table 1 shows Cu, Zn-SOD and Mn-SOD activities in different tissues of rat measured by the pyrogallol method. The highest activity is present in the liver, which is followed by adrenal gland and kidney. SOD activities of brain and pancreas are low. In separate experiments it was observed that the mitochondrial

Adrenal gland	804 ± 90	13·96 ± 1·14	2·79 ± 0·44
Kidney	750 ± 80	8·95 ± 0·69	2·00 ± 0·48
Heart	372 ± 30	3·47 ± 0·36	2·00 ± 0·17
Lung	267 ± 40	3·50 ± 0·61	1·50 ± 0·48
Brain	145 ± 20	1·90 ± 0·56	0·60 ± 0·19
Pancreas	140 ± 20	1·78 ± 0·28	0·55 ± 0·20

Each result is the average (±SD) of results from 8 young male rats (Charles Foster strain) of body weight between 175 and 250 g. SOD was assayed by the pyrogallol autoxidation method.

fraction separated at 8,500 g for 15 min by differential centrifugation of liver and kidney homogenates in 0·25 M sucrose contained almost all the Mn-SOD activity.

Table 2 shows the SOD activities in different tissues of rats of different ages, measured by the pyrogallol method. The activity was markedly low in foetus and new-born rat tissues. There was a slow but steady increase of SOD activity with postnatal development. The value increased with age and attained maximum at about 3–4 months after which it remained more or less constant up to the end of the study, about 9 months. The increase of SOD activity in rat tissues during postnatal development is probably due to an effect of exposure of the animals to atmospheric oxygen. However, a different picture was obtained in the case of erythrocyte SOD. The value was highest in 1-day-old rat and gradually decreased up to the age of 22 days and remained more or less constant up to the age of 300 days. The high erythrocyte SOD value at birth is probably to protect the haemoglobin of the new-born from oxygen toxicity.

Table 2. SOD activities at different ages in different tissues of rat.

Age (days)	Av. body weight (g)	SOD activity			
		Liver (unit/mg protein)	Lung (unit/mg protein)	Kidney (unit/mg protein)	Erythrocyte (unit/g Hb)
0[a]	6·0	1·95 ± 0·07	1·45 ± 0·08	1·40 ± 0·06	—
1[a]	6·5	2·09 ± 0·10	1·50 ± 0·10	1·40 ± 0·06	1109 ± 45·00
3	8·0	2·10 ± 0·10	1·50 ± 0·11	1·45 ± 0·05	1087 ± 43·00
5	10·0	2·50 ± 0·11	1·56 ± 0·13	1·91 ± 0·12	1065 ± 40·00
7	13·0	3·00 ± 0·27	1·60 ± 0·24	2·23 ± 0·16	1044 ± 44·00
12–14	18·0	4·65 ± 0·36	1·65 ± 0·25	3·05 ± 0·37	993 ± 47·00
20–22	26·0	7·65 ± 0·44	1·71 ± 0·20	4·69 ± 0·20	758 ± 32·00
30–35	36·0	13·25 ± 0·81	3·03 ± 0·32	9·31 ± 0·39	762 ± 30·00
60–65	95·0	16·95 ± 0·98	4·05 ± 0·43	10·85 ± 0·46	766 ± 31·00
90–110	145·0	23·90 ± 1·35	4·00 ± 0·42	10·90 ± 0·47	771 ± 35·00
120–130	195·0	24·28 ± 1·40	4·95 ± 0·52	10·89 ± 0·47	776 ± 34·00
270–300	400·0	24·00 ± 1·38	3·91 ± 0·40	10·95 ± 0·48	780 ± 33·00

Each result is the average (±SD) of results from 8 young male albino rats.
[a]Foetuses of 18 days gestation period were used; 24 rats were taken.

Table 3 shows the Cu, Zn-SOD and Mn-SOD activities in human cancer tissues measured by the pyrogallol method. The results for two samples of liver cancer and one each of oesophagus and gall bladder cancer, in contrast to those obtained for animal cancers (Oberly and Buettner, 1979) indicate that Cu, Zn-SOD is markedly low, while Mn-SOD is unaltered. Low values for Cu, Zn-SOD activity have been obtained in rabbit liver bearing tumour (Takada *et al.*, 1982), human kidney cancer (Westman and Marklund, 1981) and in other human cancer tissues (Sykes *et al.*, 1978).

Table 3. Cu, Zn-SOD and Mn-SOD activities in human cancer tissues.

Subject	Tissue		Cu, Zn-SOD (unit per g tissue)	Mn-SOD (unit per g tissue)
1	Liver	Normal	3062	754
2	Liver	Normal	3350	850
3	Liver	Normal	2860	700
4	Liver	Normal	3000	750
5	Liver	Cancer	1520	710
		Adjacent normal	2925	725
6	Liver	Cancer	1412	680
		Adjacent normal	2720	710
7	Oesophagus	Cancer	trace	170
		Adjacent normal	226	184
8	Gall bladder	Cancer	trace	192
		Adjacent normal	240	216

Samples of human liver, gall bladder and oesophagus were obtained through a local hospital from individuals subjected to laparotomy and thoracic surgery. The tissues were collected and stored in ice within 5 min after surgery, and SOD assay was performed the same day.

The pyrogallol method can be used for the assay of Fe-SOD in *E. coli* extract. Table 4 shows that 69% of the total SOD activity is Mn-SOD and 31% Fe-SOD.

Table 4. Mn-SOD and Fe-SOD activities in *E. coli* extract.

Total SOD activity[a]	Mn-SOD activity[a]	Fe-SOD activity[a]
$21 \cdot 05 \pm 0 \cdot 67$	$14 \cdot 51 \pm 0 \cdot 50$	$6 \cdot 54 \pm 0 \cdot 17$

[a]Units/mg protein.
E. coli strain B was used.

The pyrogallol method can also be used to identify SOD on gels after polyacrylamide gel electrophoresis. Figure 3 shows the achromatic zone obtained with Tsuchihasi extract of rat erythrocyte haemolysate, which disappeared after treatment with 2 mM KCN solution, indicating the presence of Cu, Zn-SOD in the
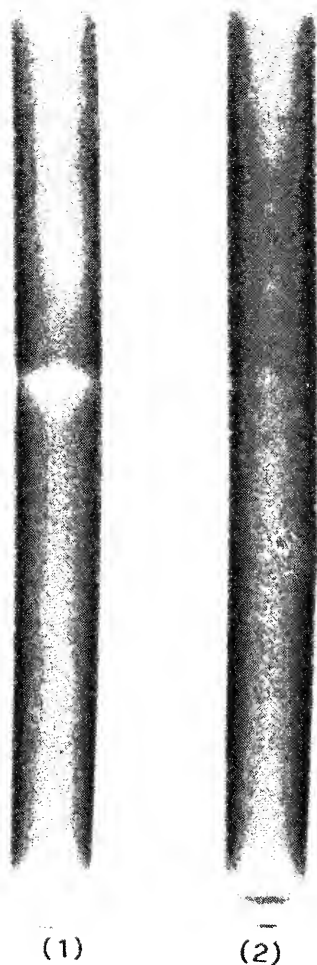
Figure 3. Activity staining for SOD on polyacrylamide electrophoretograms of Tsuchihasi extract of rat erythrocyte haemolysate. Samples equivalent to one pyrogallol unit of SOD were applied to 10% gels. (1) Rat erythrocyte Tsuchihasi extract; (2) same as (1) except that the gel was soaked in the NBT solution containing 2 mM KCN. Similar results were obtained with pure bovine erythrocyte SOD or rat liver 77,000 g soluble supernatant.

Acknowledgements

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Expression from symbiotic promoters of *Rhizobium meliloti* in *Azotobacter vinelandii* and *Azospirillum brasilense*

SARASIJA HOARE and H. K. DAS*

Genetic Engineering Unit, School of Environmental Sciences, Jawaharlal Nehru University, New Delhi 110 067, India

Abstract. In *Rhizobium meliloti*, the promoter P1 of the *nif HDK* operon, and also the promoter P2, have earlier been shown to be active in the bacteria present in alfalfa root nodules, but not in the bacteria grown aerobically in culture. Here we have looked at the expression from P1 and P2 in two non-symbiotic nitrogen-fixing bacteria, *Azotobacter vinelandii* and *Azospirillum brasilense*, using constructions in which the promoters are fused upstream of the β-galactosidase gene. The promoter P1, but not P2, is active in *A. vinelandii*, while neither P1 nor P2 is active in *Azospirillum brasilense*.

Keywords. *Rhizobium meliloti*; *Azotobacter vinelandii*; *Azospirillum brasilense*; P1 and P2 promoters.

Introduction

In *Klebsiella pneumoniae*, a free-living anaerobic nitrogen-fixing bacterium and possibly the most exhaustively studied, 17 *nif* genes (the genes for nitrogenase and other ancillary genes) are clustered in 7 or 8 operons (Dixon, 1984). The *nif H, D, K* genes, which code for the polypeptides of the enzyme nitrogenase, are present in one such operon. Another operon contains two genes, *nif L* and *nif A*. The gene *nif A* codes for a protein that acts as an obligatory positive regulatory factor for promoters of all other *nif* operons. The *nif L* is thought to code for a negative regulatory factor (Dixon, 1984).

The *nif* region of *Rhizobium meliloti* contains several operons that are specifically expressed during symbiosis in the root nodules of alfalfa (Corbin *et al.*, 1982, 1983; Ruvkun *et al.*, 1982). The promoter P1 controls the expression of the operon containing the nitrogenase structural genes. The promoter P2, located approximately 1·9 kb away from P1, operates in an opposite direction and controls the *fix ABC* operon (Corbin *et al.*, 1983). The gene *nif A* is situated approximately 4 kb downstream of the *fix ABC* operon and is controlled by its own promoter P nif A (Szeto *et al.*, 1984; Kim *et al.*, 1986). The equivalent of the gene *nif L* has not yet been detected.

Lac gene fusions of the promoters P1 and P2 have been constructed and it has been found that both the promoters in these constructs give only minimal expression in vegetative cells of *R. meliloti*. A large increase of expression was, however, observed in root nodules of alfalfa plants infected with *R. meliloti* containing both the constructs (Better *et al.*, 1985). A similar observation was made with the P nif A: : *lac Z* fusion (Kim *et al.*, 1986). On the other hand, it has been shown that in *Escherichia*

E. coli was also found to be dependent on the presence of a functional *ntr A* gene (Sundaresan *et al.*, 1983b). A P2: : *lac Z* fusion has not been tested.

These experiments provide some clues, but do not give any detailed insight into the mechanism by which the *nif* gene promoters are activated in the nodules. The *E. coli* system is more of an idealized model and is devoid of the intricate control network consisting of positive and negative regulatory factors to which the expression of the *nif* genes might be subjected in a diazotroph.

In the case of symbiotic nitrogen fixation by *R. meliloti*, the plant nodules could provide an atmosphere (low oxygen) or an activator protein essential for expression from the promoter P nif A. The plant product could also be involved in the neutralization of a negative control element elaborated in the *nif* complex that would inhibit expression from all the *nif* promoters. It would thus be of interest to study how the promoters P1 and P2 behave in the non-symbiotic and aerobic nitrogen-fixing bacterium *Azotobacter vinelandii* and the micro-aerophilic nitrogen-fixing bacterium *Azospirillum brasilense*.

Materials and methods

Bacteria and plasmids

The bacterial strains and plasmids used in this work are described in table 1.

Culture of bacteria

E. coli was cultured by shaking in LB medium at 37°C. *A. vinelandii* was cultured by shaking in modified Burk's nitrogen free (BNF) medium (Wilson and Knight, 1952) at 30°C. This medium was supplemented with 0·1% ammonium acetate. Ammonium acetate was replaced with 0·25% monosodium glutamate, wherever mentioned. Cells were harvested at mid-log phase. For growth under micro-aerophilic condition, 0·05% agar was added and the cells grown in stationary culture.

Antibiotics

Tetracycline (Tc) was used at 10 μg/ml, chloramphenicol (Cm) at 25 μg/ml and kanamycin (Kn) at 50 μg/ml.

Bacterial conjugation

The plasmids were transferred into *A. vinelandii* or *A. brasilense* from *E. coli* HB101 by conjugation, using pRK2013 as helper plasmid in a triparental mating system (Ditta *et al.*, 1980).

Assay of β-galactosidase activity

The cells were lysed by ultrasonic treatment for 1 min in an MSF Ultrasonic

(1959). Protein was determined by the method of Lowry et al. (1951).

Results and discussion

All the plasmids used in this study, except pRK2013, have the RK2 replication origin. In earlier studies (Phadnis and Das, 1987, and unpublished data) these plasmids have been found to be stable in both *A. vinelandii* and *A. brasilense*. The plasmids were transferred from *E. coli* HB101 by conjugation. *A. vinelandii* and *A. brasilense* have negligible resident β-galactosidase activity under the conditions of this study. Plasmid constructions (table 1) have been used in which the symbiotic

Table 1. Bacterial strains and plasmids used.

Bacterial strain/plasmid	Description	Reference
Escherichia coli HB101	*pro, leu, thi, lac Y, Str', end A. hsd R. hsd M*	Boyer and Roullard-Dussiox (1969)
E. coli TB1	*ara, △ (lac pro A, B), rsp L, φ 80, lac Z, △ M15, hsd R, hsd M*	G. Ditta (unpublished data)
Azotobacter vinelandii UW	Non-gummy derivative of wild type	Shah et al. (1973)
Azospirillum brasilense 7000 ATCC 29145	Wild type	Tarrand et al. (1978)
pGD926	Vector for translational fusion with β-galactosidase, Tc'	Ditta et al. (1985)
pMB210	P1 : β-gal fusion in pGD926, Tc'	Better et al. (1985)
pMB1107	pMB210△P1, Tc'	Better et al. (1985)
pMB211	P2 : β-gal fusion in pGD926, Tc'	Better et al. (1985)
pDFGD1	pMB211△P2, Tc'	Bette. et al. (1985)
pGD499	β-Galactosidase gene under the control of promoter for neomycin phosphotransferase, Tc'	Ditta et al. (1985)
pRK2013	Helper plasmid for conjugation, Kn'	Figurski and Helinski (1979)

promoters P1 and P2 from *R. meliloti* have been fused upstream of the β-galactosidase gene, so that the activity of the promoters can be assayed by the amount of the enzyme β-galactosidase formed. The construction containing the β-galactosidase gene under the control of the neomycin phosphotransferase promoter has been used as positive control. Both *A. vinelandii* and *A. brasilense* have been found to acquire resistance to Kn and neomycin when suitable plasmids containing the neomycin phosphotransferase gene are in these cells, suggesting that the relevant promoter is operational.

Studies with A. vinelandii

The results are presented in table 2. It is obvious from the results that the symbiotic promoter P1 from *R. meliloti* is operational in *A. vinelandii* when the cells are

Table 2. β-Galactosidase activity in *A. vinelandii* containing various plasmids.

| Strain (plasmid) | Character | Specific activity of β-galactosidase (nmol min⁻¹ mg protein⁻¹) | |
		Grown in BNF medium plus NH_4 acetate	Grown in BNF medium
UW (pGD926)	Control: β-galactosidase gene with no promoter	4	8
UW (pGD499)	Positive control: β-galactosidase gene under neomycin phosphotransferase promoter	205	288
UW (pMB210)	β-Galactosidase gene under *R. meliloti* P1 promoter	87	348
UW (pMB1107)	Control for pMB210: β-galactosidase gene present, but P1 promoter deleted	99	99
UW (pMB211)	β-Galactosidase gene under *R. meliloti* P2 promoter	15	17
UW (pDFGD1)	Control for pMB211: β-galactosidase gene present, but P2 promoter deleted	76	82

cultured aerobically without any association with plant factor(s) in the absence of ammonium salts.

It appears that the product of the equivalent of the gene *nif A* from *A. vinelandii* is capable of activation of the *R. meliloti* promoter P1 even under aerobic conditions. Any additional protein factor from the plant is not essential. The increase in expression activity over the control, however, is just about 4-fold, which is not quite comparable to the situation in *R. meliloti*. It is possible that anaerobic conditions inside the nodule are more conducive to expression from P1.

It is obvious that expression from the promoter P2 is controlled differently compared to expression from P1. It could need a second factor, possibly from the plant, along with a functional *nif A* product. That is why the promoter P2 does not function in *A. vinelandii*, and, in fact, acts somewhat like an inhibitor of read-through transcription from the vector (table 2).

Table 3. β-Galactosidase activity in *A. brasilense* 7000 containing various plasmids.

| Plasmid | Specific activity of β-galactosidase (nmol min⁻¹ mg protein⁻¹) | | | |
	Shake culture in Okon medium* plus NH_4Cl	Shake culture in Okon medium plus NH_4Cl, washed and incubated in Okon without NH_4Cl for 1 h	Shake culture in Okon plus sodium glutamate	Stationary culture in Okon plus sodium glutamate plus 0·05% agar (micro-aerophilic condition)
pGD926	7	2	5	8
pGD499	51	24	80	40
pMB210	50	13	47	52
pMB1107	54	24	39	49

Neither the promoter P1 nor P2 seems to function in *A. brasilense*. Promoter activity was tested under different conditions of aerobic as well as microaerophilic cell growth (table 3). *A. brasilense* can reduce acetylene (also fix N_2) under microaerophilic condition. It appears that *nif* promoters in *A. brasilense* are regulated by factors that have no effect on *R. meliloti* promoters.

Acknowledgements

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g of ferredoxin I gene from *Azotobacter vinelandii* using synthetic
ucleotide probes

G. P. DIMRI, K. B. ROY* and H. K. DAS**

Genetic Engineering Unit, School of Environmental Sciences, and *Centre for Biotechnology, Jawaharlal Nehru University, New Delhi, 110 067, India

Abstract. Two synthetic oligonucleotide probe mixtures, whose sequences were inferred from two separate stretches of amino acids, one closer to the carboxy terminal and the other closer to the amino terminal, of ferredoxin I protein of *Azotobacter vinelandii*, were used to select ferredoxin I gene clones from a cosmid gene library of *Azotobacter vinelandii*. Restriction analysis revealed that 7 out of 10 selected clones were of the same type. All these clones were found to hybridize with *fixABCX* genes of *Rhizobium meliloti*.

Keywords. Ferredoxin; *Azotobacter vinelandii*; *fix* genes; nitrogenase; Fe–S cluster.

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biological functions require auto-oxidizable low-potential ferredoxins and
oxins. The role of ferredoxins and flavodoxins in nitrogenase linked
n transport is well documented (Haaker, 1986). Ferredoxins are classified on
sis of their Fe–S clusters. *Azotobacter vinelandii*, an aerobic diazotroph,
ns, in addition to ferredoxin II (Shethna *et al.*, 1968), which is a 2Fe–2S
, another unique ferredoxin containing a 7Fe–7S cluster (Johnson *et al.*,
This can split into 4Fe–4S and 3Fe–3S clusters. The physiological significance
unusual ferredoxin is not clear. It has been suggested that this protein might
iired in aerobic nitrogen fixation in *A. vinelandii* (Kennedy and Toukdarian,
Earl *et al.*, 1987). It has been found that the *fixA, B* and *C* genes of
hizobium japonicum, which are essential for nitrogen fixation in the free-living
nave counterparts in *A. vinelandii* (Gubler and Hennecke, 1986). Here we
the cloning of ferredoxin I gene of *A. vinelandii* by screening a gene library
synthetic oligonucleotide probes. Our observations suggest that the *fixABC*
rpart in *A. vinelandii* might be the gene encoding ferredoxin I and the gene(s)

als and methods

library of *A. vinelandii* in the cosmid pHC79 was constructed. DNA from this
was transduced into *Escherichia coli* DH1 (Hanahan, 1983). Plasmid pDC2
ning *fixABC* operon of *Rhizobium meliloti* was obtained from G. Ditta,
sity of California, San Diego, USA.
oli was grown in LB, as described by Miller (1972). Antibiotics used were:
llin, 50 µg/ml, and tetracycline, 10 µg/ml.

Plasmid isolation, restriction analysis, agarose gel electrophoresis and other techniques were as described in Maniatis *et al.* (1982).

Results and discussion

Design and synthesis of the oligonucleotide probes and their labelling

The amino-acid sequence of ferredoxin I from *A. vinelandii* has been published (Howard *et al.*, 1983). From this sequence two short regions, one close to the carboxy terminal and another close to the amino terminal, were selected for the synthesis of the corresponding oligonucleotide mixtures. Selection was based on minimum codon degeneracy in the oligonucleotides. The amino-acid sequence and deduced nucleotide sequence of each are presented in figure 1. These two 17-mer oligonucleotide probe mixtures were synthesized in the Pharmacia Gene Assembler using N,N-diisopropylphosphoramidite chemistry. After synthesis, the resin-bound oligomer was deprotected at the 5'-end. Oligonucleotides were cleaved from resin and deprotected with concentrated NH_4OH at 55°C for 16 h and separated from smaller molecular weight impurities on a Nap 10 column. The fragments were finally purified by FPLC using RPC column and 5–35% acetonitrile gradient in 0·1 M triethyl ammonium acetate buffer (pH 7). Synthesis was done on 0·2 μM scale with average coupling efficiency of $96 \pm 1\%$ in each step. These were 5'-end-labelled with [γ-^{32}P]-ATP (New England Nuclear, USA, 3000 Ci/mmol) using polynucleotide kinase following the instructions of the supplier. Colonies containing the recombinant plasmids of the library were grown at a density of 1000–5000 per Petri plate and transferred onto nitrocellulose membrane (Schleicher and Schuell, BA85). The cells were lysed and the DNA denatured and hybridized to the labelled probes according to Mason and Williams (1985). Ten of the colonies that gave positive signal with both probes were purified. Plasmids were isolated from cells grown out of these colonies and digested with restriction endonucleases *Bgl* II, *Eco* RI and *Pst* I (figure 2). The clones (40–45 kb) were found to be of 4 kinds, and 7 clones were of one kind.
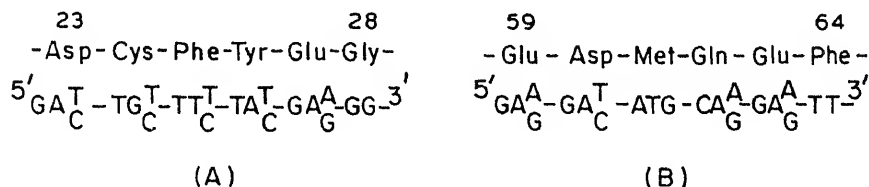
Figure 1. Sequences of two stretches of amino acids of ferredoxin I from *A. vinelandii* and the sequences of the oligonucleotide probe mixtures that have been synthesized. **A.** Sequence near the amino-terminal end. **B.** Sequence near the carboxy-terminal end.

Homology of DNA in the cosmid clones with fixABC genes
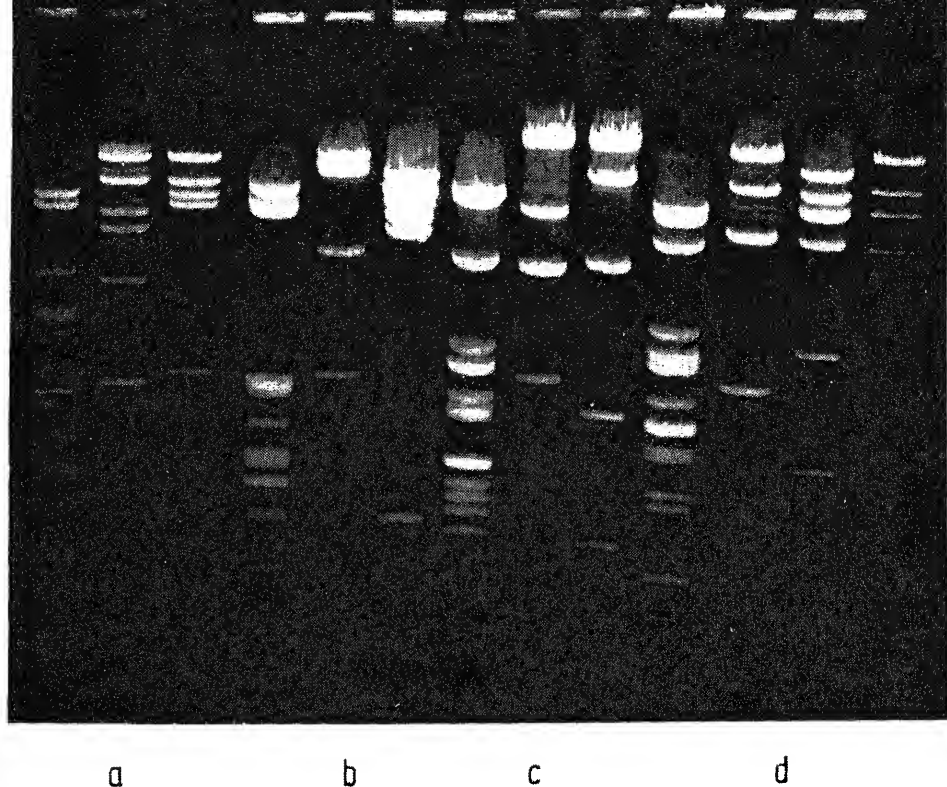
Figure 2. Restriction analysis of DNA from cosmid clones hybridizing to oligonucleotide probes. a, b, c and d are the 4 types of clones. Type c represents 7 cosmid clones, while a, b and d represent one clone each. Lane 1, DNA digested with *Pst* I; lane 2, DNA digested with *Bgl* II; lane 3, DNA digested with *Eco* RI; lane 4, lambda DNA digested with *Hin* dIII (size marker).

described by Rigby *et al.* (1977). DNA transfer onto nitrocellulose paper, hybridization, washing, etc. were carried out as described by Southern (1975) and Maniatis *et al.* (1982). *Eco* RI-digested DNA from all 4 types of clones was electrophoresed in agarose gel and transferred onto cellulose nitrate membrane according to Southern (1975). Hybridization of the DNA bands with labelled *fixABC* probe was then carried out according to Maniatis *et al.* (1982). Hybridization conditions were of high stringency. Hybridization was carried out in 6XSSC, 5XDenhardt, 0·5% SDS at 65°C overnight. The first washing was with 2XSSC, 0·1% SDS for 15 min at 25°C, and the final washing was with 0·1XSSC, 0·5% SDS for 2 h at 65°C. All 4 cosmid clones hybridized to this probe (figure 3). Gubler and Hennecke (1986) have reported a ~7 kb *Eco* RI fragment from genomic DNA of *A. vinelandii* hybridizing to *fixA*, *B* and *C* probe from *B. japonicum*. In two types of cosmids one band corresponded to this size, while in the other two it was different.
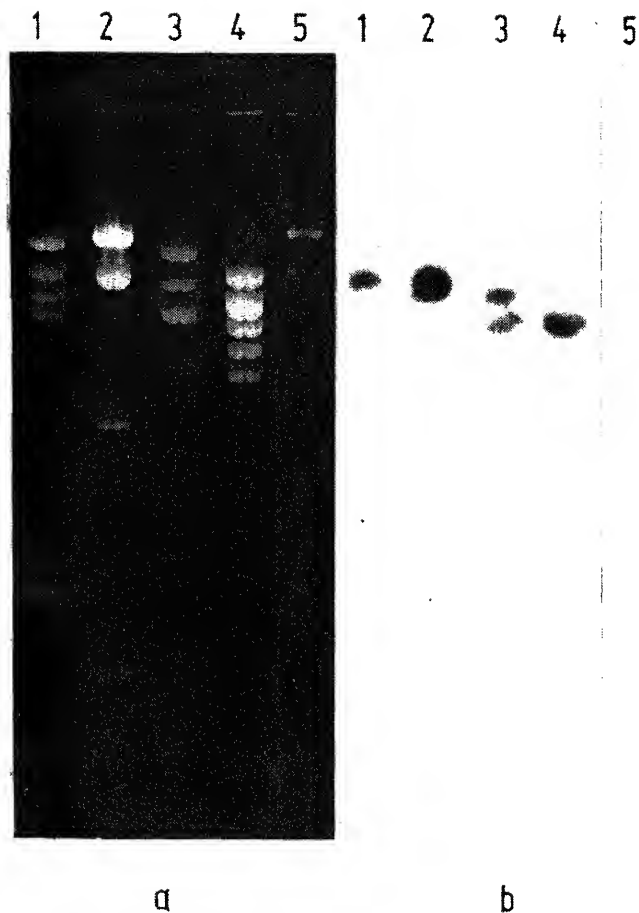
Figure 3. Hybridization of *fixABCX* probe from *R. meliloti* with DNA from the cosmid clones containing ferredoxin I-like sequences. **a.** Lane 1, *Eco*RI digested DNA from type a clone; lane 2, *Eco*RI digested DNA from type c clone; lane 3, *Eco*RI digested DNA from type d clone; lane 4, *Eco*RI digested DNA from type b clone; lane 5, lambda DNA digested with *Hin*dIII as size marker and negative control. **b.** Autoradiogram after Southern blot and hybridization.

the vector (this region also had ampicillin resistance). In the other two clones, one more band lighted up. We do not know whether it was due to some rearrangement or was because of a new region homologous to these genes. Recently, it has been reported that *fixABC* operon of *R. meliloti* has another gene *fixX* (Earl *et al.*, 1987). Nucleotide sequence and predicted amino-acid sequence of this gene were found to be more than 60% homologous with the ferredoxin I sequence. Since a part of the *fixX* gene is also contained in the probe we have used, the hybridizing region in the

ysiological significance of the unusual clustering of Fe–S centres in ferredoxin

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cular interactions between ribosomal proteins—An analysis of
), S7–S19, S9–S19 and S7–S9–S19 interactions*

V. PRAKASH

Biophysical Chemistry Unit, Food Chemistry Department, Central Food Technological
Research Institute, Mysore 570 013, India

Abstract. Ribosomal proteins S7, S9 and S19 from *Escherichia coli* have been studied by
the sedimentation equilibrium technique for possible intermolecular interaction between
pairs of proteins as well as in a mixture of 3 proteins. The proteins were isolated to a
purity greater than 95% and were characterized in the reconstitution buffer. It was
observed that none of the proteins has a tendency to self-associate in the concentration
range studied in the temperature range 3–6°C. Protein S9 behaves differently in the
presence of other proteins. Analysis of the sedimentation equilibrium data for S7–S9, S9–
S19 and S7–S9–S19 complexes revealed the need for considering the presence of a
component of higher molecular weight in the system along with the monomers and their
complexes to provide a meaningful curve-fitting of the data. Proteins S7 and S19 were
found to interact with an equilibrium constant of association of $3 \pm 2 \times 10^4 \, M^{-1}$ at 3°C
with a Gibbs free energy of interaction $\Delta G°$ of -5.7 kcal/mol. These data are useful for the
consideration of the stabilization of the 30S subunit through protein–protein interactions
and also help in building a topographical model of the proteins of the small subunit from
an energetics point of view.

Keywords. Ribosomal proteins; S7, S9 and S19; interaction; equilibrium constant; 30S
ribosome; free energy of interaction.

uction

bosome of *Escherichia coli* has been the centre of interest for a number of
s from both the functional and physical points of view. Several attempts have
nade to understand the topography of the 30S subunit of the ribosome from
st assembly map of Mizushima and Nomura (1970) using various techniques
esty and Kramer, 1985) such as chemical crosslinking (Bickle *et al.*, 1972;
; and Flaks, 1972; Shih and Craven, 1973), chemical modification of the
is (Craven and Gupta, 1970; Huang and Cantor, 1972), fluorescence energy
er studies (Huang *et al.*, 1975), fragmentation studies (Miller and Sypherd,
Morgan and Brimacombe, 1973; Schendel *et al.*, 1972), electron microscopy
is of antibody-crosslinked structures (Tischendorf *et al.*, 1974; Lake and
a, 1975), neutron scattering studies (Engleman *et al.*, 1975; Langer *et al.*, 1978)
nall-angle X-ray scattering studies (Osterberg *et al.*, 1976; Paradies and Franz
Laughrea and Moore, 1977).

sedimentation equilibrium. The numerical techniques involved are discussed in the earlier references (Rohde *et al.*, 1975; Rohde and Aune, 1975; Aune and Rohde, 1977; Aune, 1977, 1978). The selection of the proteins has been narrowed down to those that have already been shown to be in proximity, behave as monomers in solution, are of functional importance and are obtainable pure in considerable quantity.

Proteins S7, S9 and S19 are of interest in the present study since S7 is one of the proteins of the 30S subunit that interact with 16S RNA (Zimmermann, 1974; Brimacombe, 1976; Garrett *et al.*, 1974) and S7, S9 and S19 have been shown to be close to each other and to the 3'-proximal region of 16S RNA (Yuki and Brimacombe, 1975; Zimmermann *et al.*, 1975; Rinke *et al.*, 1976). The proteins S7, S9 and S19 have been shown to be part of a ribonucleoprotein fragment from the 30S subunit (Morgan and Brimacombe, 1973). The proteins S9 and S19 have been shown to enhance the binding of S7 to 16S RNA (Nomura and Held, 1974). The protein S7 is also the rate-limiting component in the assembly of S6, S7, S8, S9 and S16 (Schlessinger, 1974). The proteins S7 and S9 have been crosslinked by several workers (Lutter *et al.*, 1972; Lutter and Kurland, 1973; Bode *et al.*, 1974; Lutter *et al.*, 1974, 1975; Sommer and Traut, 1975; Expert-Bezancon *et al.*, 1977; Langer *et al.*, 1978). Further, Bickle *et al.* (1972) have crosslinked proteins S6, S7 and S9 using dimethyl suberimidate. Antibody to S19 inhibits Fmet-tRNA binding according to Traut *et al.* (1974). In addition, Rummel and Noller (1973) have clearly shown that protein S19 is protected from trypsin digestion by the prior binding of tRNA to the ribosome.

These data aid in visualizing a stable topographical model of the proteins of the 30S subunit of *E. coli* from an energetics point of view. In this paper the isolation and characterization of proteins S7 and S19 and the interaction between the proteins S7 and S9, S7 and S19, and S9 and S19, and between the 3 proteins in the ternary complex S7–S9–S19 are reported.

Materials and methods

Isolation of S7, S9 and S19

The 30S and 50S ribosomal subunits were isolated from *E. coli* MRE 600 cells as described by Rohde *et al.* (1975). The individual fractions containing a fair amount of S7, S9 and S19 were obtained by chromatography on a phosphocellulose column using the procedure of Hardy *et al.* (1969) and Rohde *et al.* (1975). The proteins S7, S9 and S19 were tentatively identified depending on their mobility in gel electrophoresis in the presence of urea and positions as described by Hardy *et al.* (1969) and Rohde *et al.* (1975).

The fraction from the phosphocellulose column containing protein S7 along with protein S3 or S16, which coelute, was loaded onto a Sepharose 4B–100 column (1·5 × 85 cm) in 6 M guanidine hydrochloride (GuHCl) containing 0·001 M 2-mer-

n (1·5 × 85 cm) in 6 M urea buffer, pH = 5·6. The protein S7, obtained pure
he above procedure, was pooled, dialysed free of salt, and lyophilized. The
geneity of the sample was judged by gel electrophoresis in the presence of urea
y a modified sodium dodecyl sulphate (SDS) gel electrophoresis (Weber and
n, 1969; Rohde et al., 1975). The gels stained with amido black were scanned
ISCO UA-5 absorbance monitor at 546 nm.

fraction containing primarily S19 also contained small quantities of S14, S15,
12 or S13. The fraction was loaded on a Sephadex G-100 column (1·6 × 76 cm)
4 urea buffer, pH 5·6. Protein S19 elutes as the major peak with two other
peaks eluting just after the void volume. The protein S19 obtained thus was
ed on the Sephadex G-100 column and obtained in 95% homogeneity. The
geneity of the protein was tested by the methods described for S7.

isolation of protein S9 has been described earlier (Prakash and Aune, 1978c).

acid analysis

acid analysis of the proteins was carried out in a Beckman Model 121
acid analyser by the method of Spackman et al. (1958). The amino acid
sis was compared with the published data of Craven et al. (1969) and
chmidt et al. (1970) and the tentative assignments made earlier were confirmed
mputing a correlation coefficient with the amino acid values available in the
ure for the above proteins.

ptivity

rptivity of the proteins was determined in an analytical ultracentrifuge (Aune
imasheff, 1971) using a synthetic boundary cell. Absorptivity at 280 nm of S7
19 was determined in 15% acetic acid at 20°C because the proteins were not
sufficient concentration in Tris–MgCl$_2$–KCl (TMK) buffer for an accurate
rement. Lysozyme was used as the calibrating protein. Absorptivity was
ed to be the same in both TMK buffer and 15% acetic acid for the purpose of
ating the concentration of the proteins.

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roteins were refolded in TMK buffer. The lyophilized proteins (0·5–0·6 mg)
dissolved in about 20 µl of 6 M GuHCl. TMK buffer, 0·5 ml, was added, and
olution was incubated at 37°C for 45 min. The solution was then dialysed
st TMK buffer at 5–6°C with several changes, the last change normally lasting
least 12 h. The dialysed samples were clarified at 24,000 g at 5°C for 30 min.
JV spectrum of the protein was then obtained. From the UV spectrum and
ptivity data, the concentration of the protein was computed. In those spectra

an epon-filled double sector synthetic boundary cell with a protein concentration yielding an absorbance of 0·5–0·6 at 280 nm in TMK buffer. The $S_{20,w}$ values were computed using a Hewlett-Packard 9810A programmable calculator. The log amplified output of the scanner phototube is digitized through an integrating voltmeter and read into the calculator. The details of the procedure adopted are discussed by Inners *et al.* (1978).

Stokes radii and related parameters: Stokes radii of the proteins were determined by gel chromatography using the procedure of Ackers (1967). A Sephadex G-100 column was used, emplyoing 0·1 M KCl TMK buffer, pH 7·36. From the Stokes radius f/f_{min} was calculated.

Molecular weight

Sedimentation equilibrium: Molecular weights (M_r) were determined by sedimentation equilibrium in TMK buffer employing a modification of the high-speed technique of Yphantis (1964). The details of the procedure have been discussed by Aune (1978). Monomer M_r were obtained in 6 M GuHCl containing 0·001 M 2-mercaptoethanol. Data were obtained at several speeds for the mixture of proteins as well as for the homogeneous samples. Plates were read on an LP-6 profile projector equipped with a Nikon stage and micrometers. The calculations of the number, weight and Z-average M_r were performed using a Hewlett-Packard 9810A programmable calculator. Curve-fitting procedures for the monomer, dimer and higher-order complexes along with the mixtures were performed in a Digital DEC-10 computer with a program written in Fortran which employs the procedures discussed previously (Aune and Rohde, 1977).

SDS-polyacrylamide gel electrophoresis: The M_r of the proteins was also obtained by the method of SDS-polyacrylamide gel electrophoresis (PAGE) as described by Weber and Osborn (1969) using standard markers. A 12·5% gel with a 1·25% crosslinking was employed and stained with Coomassie brilliant blue.

Results and discussion

The proteins S7 and S19 were tentatively identified based on mobility in gel electrophoresis in the presence of urea and gel chromatography profiles. Confirmation of the identification was based on the correlation coefficients computed from a comparison of their amino acid composition with that already available in the literature (Craven *et al.*, 1969; Kaltschmidt *et al.*, 1970).

The absorptivity at 280 nm of proteins S7 and S19 was found to be $0·72_3$ ml mg^{-1} cm^{-1} and $0·55_5$ ml mg^{-1} cm^{-1}, respectively in 15% acetic acid at 20°C.

The individual proteins S7, S9 and S19 were characterized in solution for minimum M_r, M_r in TMK buffer, $S_{20,w}$ value, Stokes radius and degree of self-

Molecular heterogeneity of a system can be quantitated by analysing data from sedimentation equilibrium experiments. In such experiments the concentration distribution of a single species or a mixture of proteins is given by

$$C(r) = \sum_i C_i(a) \left[\exp \left\{ M_i (1 - \bar{v}\rho) w^2 (r^2 - r_a^2)/(2RT) \right\} \right], \tag{1}$$

where C is the concentration of the ith component at the meniscus, M_i the M_r, $\bar{v}$ is the partial specific volume, ρ is the solution density, w is the angular velocity, r the radial position and r_a the radial position at meniscus, R the gas constant and T the absolute temperature.

The weight average M_r of S7 over the whole cell was computed to be $16,000 \pm 500$. Figure 1a presents a plot of fringe displacement (in micrometres) *versus* radial position, with a maximum displacement of nearly 900 μm. An earlier report from this laboratory from a different preparation of S7 provided M_r of $20,100 \pm 1000$ in TMK, $18,700 \pm 1000$ in GuHCl and $21,000 \pm 2100$ by SDS–PAGE (Rohde and Aune, 1975). The variability in the M_r observed may be because the strains of *E. coli* from which the protein was purified are different in the two studies. S7 in this study has been isolated from *E. coli* MRE 600 cells and the strain used by Rohde and Aune (1975) was *E. coli* B. The sedimentation equilibrium data for S7 in TMK buffer at 3°C were subjected to curve-fitting procedures. The data fit very well to a system with monomer alone with an average residual of 8 μm. The average residual R is defined by

$$R = \sum_{i=1}^{N} \left[|\delta_i|/(N - S - 1) \right], \tag{2}$$

where N is the number of data points, S is the number of species in the system, and
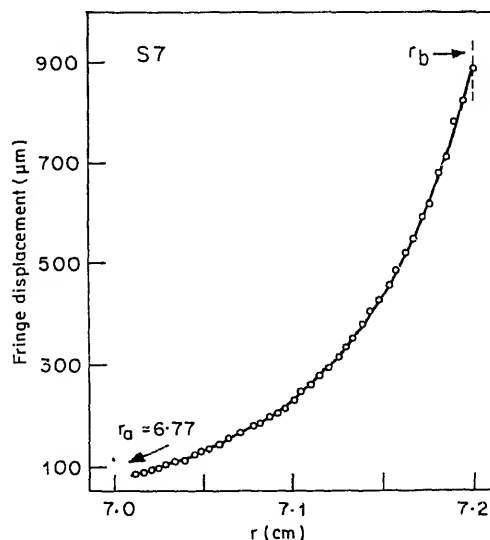
σ is the absolute value of the residual at each point, the residual itself being the difference between the calculated value based on M_r and meniscus concentration and the experimental value. The solid line in figure 1a indicates a monomer fit through the experimental data points. It is clear that no higher-order aggregate is present in significant amounts.

The M_r of S19 determined from sedimentation equilibrium data was $11,000 \pm 700$ in TMK buffer at 3°C. Figure 1b shows a plot of fringe displacement *versus* radial position for S19 with a displacement of 1100 μm towards the bottom of the cell. From SDS–PAGE a M_r of $13,400 \pm 1000$ was obtained. The sequence M_r of S19 from *E. coli* K has been reported to be 10,299 (Yaguchi and Wittmann, 1978). The data obtained here from the sedimentation equilibrium experiment is in excellent agreement with the sequence M_r. The sedimentation equilibrium data indicate that the protein exhibited monomeric behaviour over the whole concentration range in the centrifuge cell, suggesting the absence of self-association under the conditions of the experiment. The curve-fitting procedures of the data indicated a monomeric species only with a M_r of 11,000. The solid line in figure 1b indicates the calculated curve for the monomer under the experimental conditions.
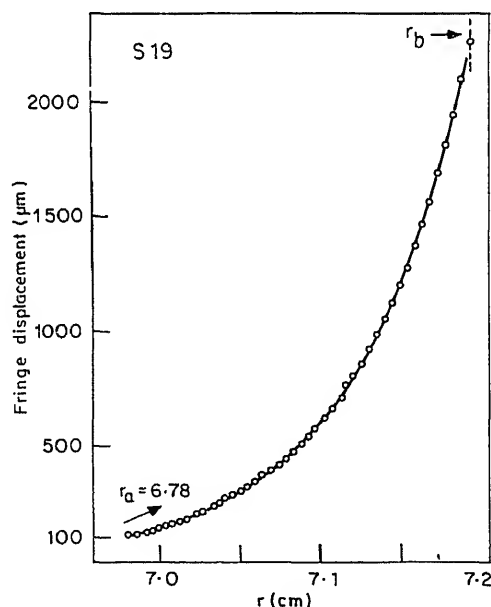
Figure 1b. Fringe displacement (μm) *versus* radial position. Sedimentation equilibrium experiment conditions S19, 0·37 mg/ml, 36,000 rpm; solid line is the best fit for a single species.

The protein S9 has been reported to have a M_r of 14,000 in TMK buffer at 3°C and is monomeric over the concentration range in the sedimentation equilibrium experiment (Prakash and Aune, 1978c). In addition to the minimum M_r and M_r in

of the dimeric and trimeric complexes.

Protein/complex	$S_{20,w}$	f/f_{min}	$R_s(\text{A}°)$	o	f/f_{min}
S7	$1·66\pm0·1$	1·35	21·2	0·58	1·27
S19	$1·04\pm0·05$	1·64	22·2	0·53	1·50
S7–S9[a]	$1·32\pm0·06$	—	—	—	—
S7–S19[b]	$1·30\pm0·24$	—	—	—	—
S9–S19[a]	$1·61\pm0·21$	—	—	—	—
S7–S9–S19[c]	$1·83\pm0·03$	—	—	—	—

[a]Ratio 1:1 by mass. [b]Ratio 1:1·4 by mass. [c]Ratio 1:1:1 by mass.

t method. Necessarily, f/f_{min} is smaller than previously reported. From both
ntation and gel chromatography it is observed that S19 appears to be an
ed molecule. The f/f_{min} value computed from both $S_{20,w}$ and R_s turns out to
er than the typical values of f/f_{min} for globular proteins, which are of the
f 1·20–1·30 in a non-denaturing solvent.
3 proteins were refolded as described under materials and methods, dialysed
GuHCl against TMK buffer, and clarified. After computing concentration,
teins were mixed in proper ratios, dialysed again against TMK buffer, and
d for both $S_{20,w}$ values and M_r distribution by sedimentation equilibrium at
speeds.

oteins S7 and S19 were mixed 1:1·4 by mass ratio in TMK buffer and data
btained from both sedimentation velocity and sedimentation equilibrium
nents. The $S_{20,w}$ value for the mixture at a total concentration of 0·5 mg/ml
lculated to be $1·30\pm0·24$ S, which is almost a weight average of the two
ers.
sedimentation equilibrium data do reveal components of M_r higher than that
er monomer from the point averages of the weight average M_r. The plot of
tural logarithm of fringe displacement versus r^2 was significantly curved
ing the heterogeneous nature of the system. Figure 1c shows a plot of fringe
ement in μm versus radial position for the mixture. The data were subjected
ve-fitting procedures and found to fit very well to a system consisting of 3
, i.e. S7, S19 and S7–S19, instead of just two non-interacting species S7 and
hese data are therefore consistent with a model where proteins S7 and S19
t with the simplest stoichiometry of 1:1. The percentage mass as complex
was calculated according to the equation

$$\% \text{ complex} = 100 \cdot \int_a^b C_3 \, dr^2 / \int_a^b C \, dr^2, \tag{3}$$

gration from the meniscus a to the bottom of the cell b, where
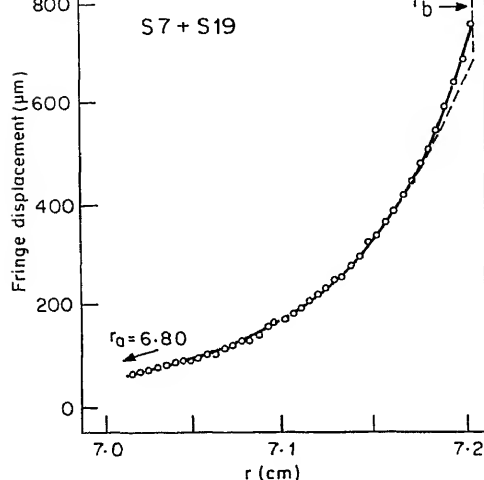
Figure 1c. Fringe displacement (μm) *versus* radial position. Sedimentation equilibrium experiment conditions 1:1·4 ratio '(0·25:0·35 mg/ml of each) of S7:S19 at 33,450 rpm at 3°C. The dashed line is the best fit for two species and the solid line is the best fit for 3 species.

is significant enough to recognize the interaction between the two proteins. The data obtained from the curve-fitting performed on sedimentation data for the S7–S19 mixture are given in table 2. These data are utilized for computing the equilibrium constant of association by the equation

$$K = \frac{M_1 M_2}{M_3} \cdot \frac{C_3(a)}{C_1(a) \cdot C_2(a)}, \tag{5}$$

where 1 and 2 represent the proteins that associate to form the complex 3 and C is the concentration at the meniscus. The detailed procedures are described by Aune

Table 2. Parameters determined from curve-fitting of sedimentation data for the mixtures S7–S19, S7–S9, S9–S19 and S7–S9–S19.

Pair	Expt. No.	R^a	R^b	Average residual (μm)	Percentage mass as complex	Equilibrium constt. of association ($\times 10^{-4}$ M)
S7–S19	1	1·4	2·2	4·7	9·0	2·61 ± 0·97
	2	1·4	2·0	10·1	13·0	4·29 ± 3·55
	3	1·4	1·8	12·4	8·0	2·08 ± 1·51
						3·00 ± 2·01[c]
S7–S9	1	1·0	1·1	11·8	15·0	1·61 ± 0·58
S9–S19	1	1·0	1·5	10·0	12·0	6·16 ± 2·0
S7–S9–S19	1	1:1:1	1:1·2:1·1	4·0	6·0	––
					(1–2% of 123,000/	

ium constant of $3 \pm 2 \cdot 01 \times 10^4$ M^{-1}, which gives a Gibbs free energy of
ion $\Delta G°$ of $-5 \cdot 7$ kcal/mol at 3°C in TMK buffer. These data support the
from crosslinking experiments that the two proteins are together, as has been
ned earlier.

oteins S7 and S9 were refolded as mentioned earlier in materials and
s and were mixed in 1:1 ratio by mass. The mixture was analysed by
ntation velocity and sedimentation equilibrium centrifugation.
$S_{20, w}$ value of the mixture was calculated to be $1 \cdot 32 \pm 0 \cdot 06$ S. The interaction
by sedimentation equilibrium experiments were performed at 3°C and figure
vs a plot of fringe displacement *versus* radial position for the mixture. Curve-
of the data was initially performed for two species, S7 and S9, only, but this
ed that a higher M_r species towards the bottom of the cell would have to be
red. The data demanded a complex of even higher M_r than just 30,100 (the
S7 and S9 M_r) to be present in the system. After several trials the data were
tted with considerably lower error (in the range of plate reading) to a system
ng of species of M_r 14,100, 16,000, 30,100 and 120,400. Further, it is to be
hat the data are also consistent with low residuals for components of M_r
16,000 and 150,000. No real distinction can be made between these two
or any other comparable combination. The typical data for the S7–S9
are given in table 2. It can be seen that a complex of M_r 120,400 must be
rated into the system in order to have meaningful curve-fitting at the
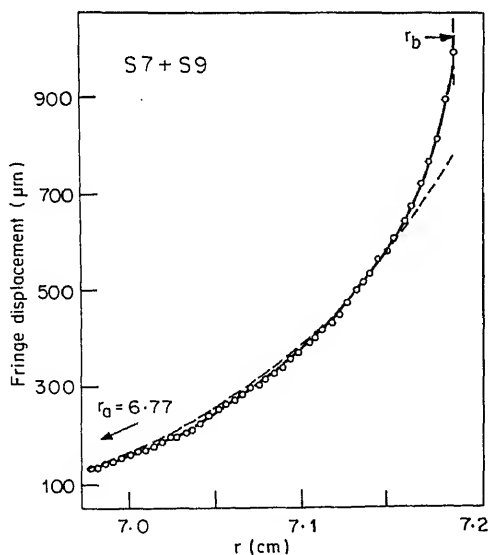
Figure 1d. Fringe displacement (μm) *versus* radial position. Sedimentation equilibrium

The two proteins S9 and S19 were refolded in TMK buffer and mixed in 1:1 ratio by mass as described under materials and methods. The mixture was analysed by sedimentation velocity and sedimentation equilibrium techniques at multiple speeds.

From table 1 it can be seen that S19 has an $S_{20,w}$ value of 1.04 ± 0.05 S. The mixture of the two proteins (total concentration ~ 0.7 mg/ml) sediments at 1.61 ± 0.21 S at 20°C. This indicates higher M_r material in the system than just the monomers themselves.

The mixture was examined by sedimentation equilibrium centrifugation employing multiple speeds only at low temperature. Figure 1e shows a plot of fringe displacement *versus* radial position for the mixture. The data were subjected to curve-fitting procedures as before. A logical analysis of the data indicated at least two possible models satisfying the given set of data. The first model predicts a system consisting of the monomers, the complex, and a hexamer of the complex of M_r 150,600. The second model, with the monomers and the hexamer of the complex of M_r 150,600, fitted the data equally well with the residuals of the curve-fit being
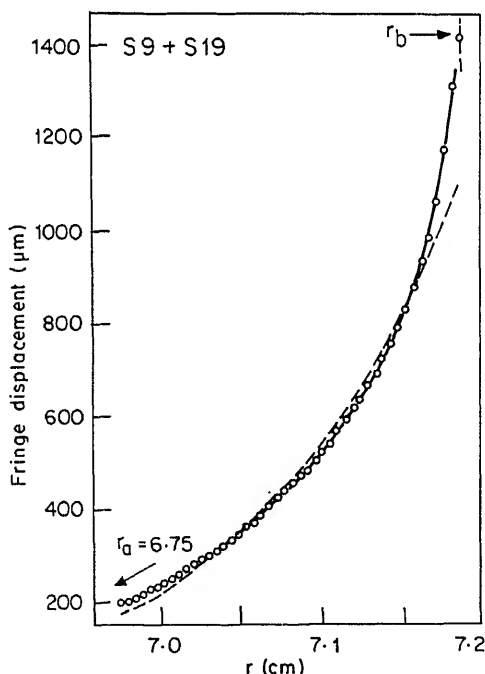
Figure 1e. Fringe displacement (μm) *versus* radial position. Sedimentation equilibrium experiment conditions 1:1 by mass (0.30 mg/ml of each) of S9:S19 at 27,690 rpm at 3°C. The dashed line is the best fit for the two species and the solid line is the best fit for the

10 μm. The data for the S9–S19 interaction are also given in table 2. They
e that S7 and S19 interact to give a complex in TMK buffer.

–S19

tion of a ternary complex was investigated by mixing S7, S9 and S19 in the
: 1 : 1 by mass and analysing the data obtained from sedimentation velocity
dimentation equilibrium experiments at several speeds.
$S_{20, w}$ value for the mixture was 1.83 ± 0.03 S at 20°C (table 1). The mixture
ialysed overnight against TMK buffer. The higher $S_{20, w}$ obtained here
tes the presence of a higher M_r complex in the system since none of the
ners has an $S_{20, w}$ value greater than 1.65 S. The result and conclusion from
periment are more of a qualitative nature because of the complexity of the
ation involved and the heterogeneity of the sample. Sedimentation
orium experiments at low temperature provided data which were then
ed by the curve-fitting procedures mentioned earlier. Figure 1f shows a plot
ge displacement *versus* radial position for the S7–S9–S19 sedimentation
orium experiment. A system with 5 species of M_r 11,000, 14,100, 16,000, 43,300
3,300 (a trimer of the complex of the monomers) provides a residual error of
and proper mass ratio. However, this extreme 5-species fit leaves the system
ning only 1–2% of the mass as a high M_r complex. Intermediate, more
ex systems with simple trimers as well as high M_r complex suggest 4% of the
nvolved in interaction. Even though the amount of the complex cannot be
d, the presence of it, even at low levels, is essential for meaningful curve-fitting
he sedimentation equilibrium data.
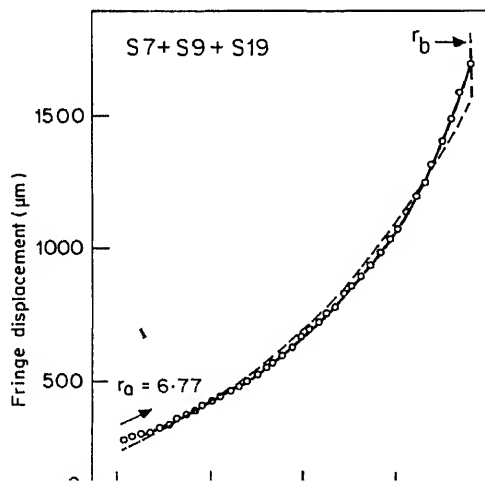
both in pairs and as a ternary combination. The nature of these complexes cannot be identified with the present data.

Table 3 gives the energy of interaction for some pairs of 30S ribosomal proteins. The data indicate that even though S7 and S19 interact to a considerable degree, the interaction is not as strong as that between S3 and S5 or between S5 and S10, but is stronger than that between S4 and S5.

Table 3. Energy of interaction for some pairs of 30S ribosomal proteins.

Pair	Interaction	$\Delta G°$ (K cal/mol)
S2–S3[a]	−	—
S3–S4[b]	+	−5·1
S3–S5[c]	+	−7·3
S4–S5[b]	+	−4·8
S4–S9[c]	+	−5·8
S4–S20[b]	−	—
S5–S10[a]	+	−7·4
S6–S18[d]	+	−6·0
S6–S21[e, f]	+	−6·6
S7–S9	+	−5·3
S7–S19	+	−5·7
S9–S19	+	−6·0
S18–S21[b]	+	−5·6

[a]Rohde and Aune (1975). [b]Aune (1977). [c]Rohde et al. (1975).
[d]Prakash and Aune (1978a). [e]Prakash and Aune (1978b).
[f]Prakash and Aune (1978c).

The results of the present studies indicate that the proteins, S7, S9 and S19 are monomeric in TMK buffer at 3°C and S9 aggregates at higher temperatures. The protein S9 behaves differently in the presence of S7 and S19. The behaviour of S9 in the presence of S4, although different, still permits an evaluation of the equilibrium constant of association (Prakash and Aune, 1978c). When S7 and S19 were mixed and the mixture analysed by sedimentation equilibrium experiments, 8–13% complex formation was observed, there were no higher-order complexes, and the energy of interaction was computed to be −5·7 kcal/mol. The presence of S9 in the system, S4–S9 (Prakash and Aune, 1978c) S7–S9, S9–S19 or S7–S9–S19, makes the complex of the proteins to assume a very high M_r. The data suggest an unusual role for the protein S9. Since the assembly process must contend with these thermodynamic states it would appear that protein S9 is a dynamic 'glue' in the 30S ribosome. It is more interesting that in order to restrict complex formation of S9 with a single protein, nature has probably placed it amidst a number of other 30S ribosomal proteins, amongst them S4, S7 and S19, with which it can complex. Compared to other models the significance of S19 in the proximity of S4, S7 and S9 is to be noted. In view of the models of Nomura and Held (1974) and Cornick and

map of the small subunit of the *E. coli* ribosome.

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Biochemical analysis as a measure of dynamic equilibrium in genomic setup during processing of tea*

PRADIP K. MAHANTA

Department of Biochemistry, Tocklai Experimental Station, Tea Research Association, Jorhat 785 008, India

Abstract. The genetic characteristic such as 'fermentability' of a tea cultivar could be utilised to obtain maximum colour/bloom during manufacture of black tea. Pigment profile analysis has been used as a tool to assess the characteristic of a black tea brew. Fine plucking and optimum processing conditions are two basic requirements in producing good quality black tea. The assamica variety is characterised by linalool content while geraniol is specific in chinary clones. The higher amounts of terpenoids improved the flavour characteristic of second flush tea of north east India in general and Darjeeling in particular. Further, the surplus fatty acid degradation products lower the quality of black tea during monsoon flush.

Keywords. Cultivar characteristic; manufacturing variations; pigment profiles; fatty acids; volatiles.

Introduction

Tea is one of the major agrocommercial products of India. It is a parennial crop and is harvested throughout the year at weekly intervals. Normally, a pluckable shoot is made of growing leaves with internodes separated by nodes, the point at which axillary bud is subtended. Plucking is one of the important practices intimately connected with generation of new shoots. The amount of growth made by the apical bud between the two successive states of dormancy is termed as 'flush'. There are 4 distinct flushes, namely, first, second, third or monsoon and the fourth or autumn flushes (Baruah, 1970). Seasonal changes are associated with plant metabolism during various flushing periods of tea. The processing conditions followed routinely by the industry to cope with the harvested crop, produced black teas which are distinct in character and different in quality. Orthodox and crush–tear–curl (CTC) are the principal catagories of black tea. Their manufacturing techniques differ considerably and have a pronounced impact on the formative and degradative patterns of various cellular components (Mahanta and Hazarika, 1985).

Sensory techniques to assess overall quality such as texture of made tea and characteristic of the brew have been useful guides but have their own limitations. Correlation of dynamic release of quality attributes such as colour and flavour during black tea processing are of special interest but is still poorly understood (Lee, 1986; Mishkin *et al.*, 1984). Catechins, and chlorophyll and its derivatives have been implicated as the colour contributing substances while carotenoids and fatty acids have been identified as responsible for flavoursome components of made tea

the factory floors.

Materials and methods

Black tea processing

Tea shoots from JTCL-340 and CNMA 33/52 (released from Tocklai) of different plucking standards such as fine and course pluckings were manufactured in the miniature factory during the seasons 1984 and 1985. The various Tocklai vegetative clones studied were Assam type (TV2), China type (TV1, 7 and 17) and Cambod type (TV9, 18 and 19). The colour and aroma compositional analyses were carried out by methods like pigment and volatile profile analyses (PPA and VFC).

Analysis

Coloured compounds: Black tea samples, orthodox as well as CTC were extracted with 40 ml 60% aqueous acetone. Filtrate (0·3 ml) containing about 20 mg extract was separated over a Sephadex LH 20 column chromatograph into 6 fractions (I–VI) known as pigment profile analysis (table 1 and figure 1). The fractions contained mostly theaflavins (TF), thearubigins (TR) along with chlorophyll and its derivatives (Hazarika *et al.*, 1984).

Volatile flavour compounds

Volatile compound extraction from orthodox and CTC teas were carried out either by simultaneous steam distillation and ether extraction (SDE) or vacuum steam distillation methods and the characterisations were done by gas chromatography and mass spectrometry (GC-MS). Volatile flavour profiles of non-terpenoids such as trans-2-hexenal and the monoterpenoids such as linalool and its derivatives and geraniol were investigated upon for various processed teas (Takeo and Mahanta, 1983; Baruah *et al.*, 1986).

Fatty compounds

Extraction and quantitation of crude lipid, chlorophyll and carotenoids were carried out throughout the plucking seasons (Hazarika and Mahanta, 1984; Mahanta *et al.*, 1985).

Fatty acid methyl ester

Crude lipid was hydrolysed in alcoholic KOH and the free fatty acids were

packed with 10% DEGS. Column temperature was programmed from 130–200°C at 4°C/min.

Results and discussion

TF, TR and chlorophyll derivatives

Black tea is known as fermented tea. The withering-rolling-fermentation and drying stages of black tea manufacture enable the leaf cells to break so that the solids of made tea could dissolve while brewing. Depending on inherited character a tea cultivar upon maceration in the orthodox rollers and CTC machine undergo fermentation in the presence of air. Atmospheric oxygen has been found to be instrumental for production of a host of coloured compounds especially, TF, TR and pheophytin etc. However, the polyphenols and/or oxido-reductase enzymes are mostly responsible for the production of golden yellow TF and reddish-brown TR as indicated in table 1 (Takino, 1972). TR are intimately connected with the colour and taste characteristics of the brewed tea. Chlorophyll and its derivatives such as pheophytin and pheophorbide have also been found to contribute towards the shade of colour of made tea. A taster judges the quality of a product from its taste and

Table 1. Characteristics of soluble products formed during rolling-fermentation.

Compound	Fractions nos	Weight (%)	Colour	Colour contribution (%)
TF	VI	0·28–1·63	Golden yellow	30
TF monogallate	VI	– do –		
TF digallate	VI	– do –		
TR, TR-1	I	5·1–14·8	Reddish brown	35
TR, TR-2	II	– do –		
TR, TR-3	III	– do –		
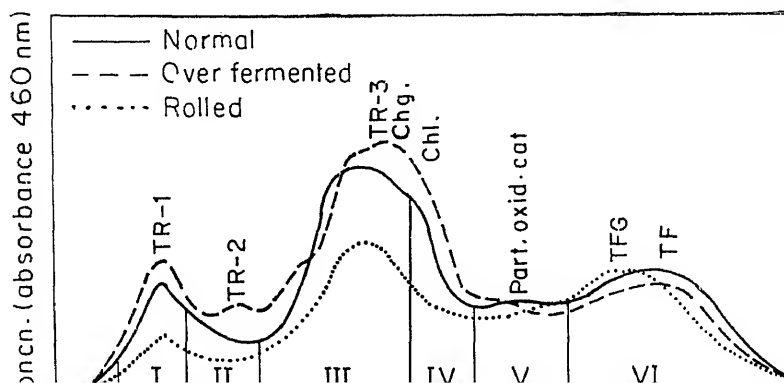
1984). From the present study pigment profile analysis could be an advantageous tool in evaluating fermentation characteristics of a brewed tea (figure 1). From the figure 1 it is clear that maximum peaks in TF and TR have been attained during optimum fermentation while in case of over fermented teas undesirable TR formations have been indicated by reduction of TF peak. TR fraction especially TR-1 can be a measure of thin or a good liquor character of the brew. The study of TR fractions can help in evaluating the rate of fermentation of a cultivar. Thus chinary clones have been found to be fast fermenting followed by Cambod and Assam clones. Chemical composition and their corresponding organoleptic quality of fine and coarse plucked and different degree of withered black teas are shown in tables 2 and 3. High fibre content and low water soluble solids appear to control the valuation in the brewed tea (Baruah *et al.*, 1986).

Table 2. Shoot fineness and chemical composition (% dry wt.) of black tea of different plucking round and tasters' quality.

	5 day	7 day	9 day	11 day
Fineness	120	100	80	60
Ash	5·95	6·15	6·16	6·15
Crude fibre	6·7	7·0	9·4	10·5
Total water soluble solids	44·36	42·47	42·50	41·44
Caffeine	4·40	4·79	4·32	3·81
TF	1·12	1·22	1·25	1·40
TR	13·56	13·98	14·19	15·74
TF/TR	0·08	0·09	0·09	0·09
Tasters' evaluation	Very good	Good	Good	Fair

Table 3. Chemical composition (% dry wt.) of orthodox black tea of different degree of withering.

Withering (%)	Total water soluble solids	Caffeine	Ash	Crude fibre
68 (Normal withered)	37·32	3·68	6·15	14·26
50 (Hard withered)	39·07	4·14	6·84	11·87
CD between 2 types of withering at 0·1% level of probability	0·38	0·35	0·31	0·26

The values are based on the average in triplicates.

Flavour volatiles

Hexenals and linalools: Lipid content in leaf tissues undergo transformation into volatile flavours during processing caused either by the hydrolysing or the oxidising action of enzymes (Mahanta *et al.*, 1985). However, the process of withering and rolling plays a vital role in the release of flavoury volatiles from non-volatile precursors (Takeo and Mahanta, 1983). Thus higher concentration of trans-2-hexenal
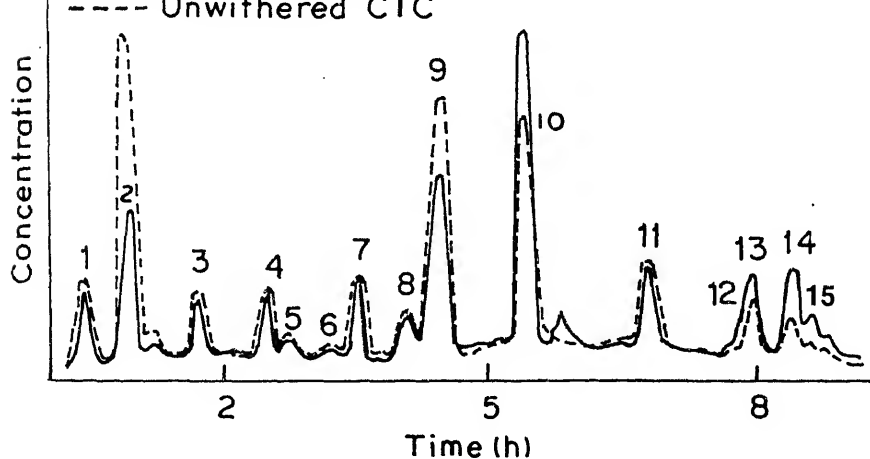
Figure 2. Difference in volatile flavour components of CTC black teas manufactured from withered and fresh leaf.
1, 1-Penten-3-ol; 2, trans-2-hexenal; 3, cis-2-penten-1-ol; 4, n-hexanol; 5, cis-3-hexenol; 6, trans-2-hexenyl formate; 7, linalool oxide (5-cis); 8, linalool oxide (5-trans); 9, benzaldehyde; 10, linalool; 11, standard; 12, methyl salicylate; 13, geraniol; 14, benzyl alcohol; 15, 2-phenyl ethanol.

(Mahanta, 1988). Assuming monoterpenes as the 'key aroma' constituents, it has been found that the aroma characteristics are also related to the genetical peculiarities of the tea plant. Linalools have been found to be characteristic of assamica clones while geraniol is characteristic of chinary clones (Takeo and Mahanta, 1983).

Figure 3 shows the difference in the volatile flavours of plain Assam and Darjeeling teas. The characteristic rosy and heavy thick flavour of Darjeeling teas may be
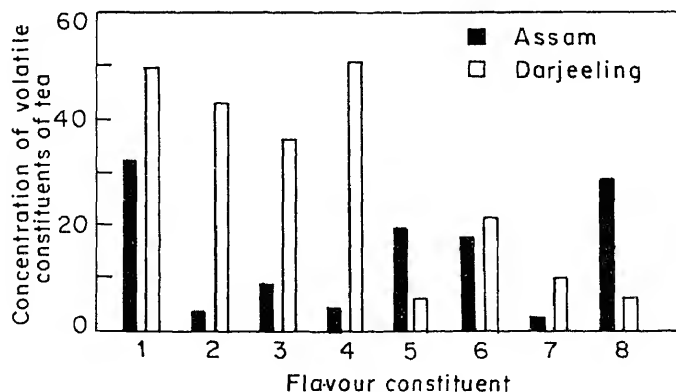
Figure 3. Difference in volatile flavour components of Assam and Darjeeling teas.
1, Linalool oxide (furanoid); 2, linalool; 3, linalool oxide; 4, geraniol; 5, benzyl alcohol; 6, 2-phenyl ethanol; 7, hexenol; 8, phenyl acetaldehyde.

attributed to the fact that the planting material originated from Keemun district of China (Yamanishi, 1981).

Coarse plucking and hard withering

It is seen that the non-terpenoids derived from fatty acid degradation increased and terpenoids decreased in coarsely plucked teas as well as in hard withered black teas (tables 4 and 5). The monoterpenes, especially linalools were found to be more in finely plucked shoots of 5 days plucking round, which progressively decreased with increased plucking interval upto 11 days under north eastern Indian conditions. Loss of terpenes in made tea has been found to develop a deleterious organoleptic quality which in turn is responsible for lower valuation of tea.

Table 4. Terpenoid and nonterpenoid ratio of different plucking standards Assam CTC tea and different elevations of Darjeeling orton tea.

Samples	Total terpenoid	Total non-terpenoid	Total VFC	Terpenoid/nonterpenoid
TR$_1$ (75% FP[a])	1·43	10·17	11·60	0·14
TR$_2$ (60% FP)	1·34	8·60	9·94	0·16
TR$_3$ (40% FP)	1·13	9·11	10·24	0·12
High elevation[b]	8·78	9·72	18·50	0·90
Mid elevation	7·13	11·46	18·59	0·62
Low elevation	7·26	8·07	15·33	0·90

[a]FP, Fine plucking.   [b]At Ging Tea Estate, Darjeeling.

Table 5. Total volatile components of black tea manufactured from different degree of withering and the terpenoid, non-terpenoid ratio.

Volatile compounds	50% withered orthodox	68% withered orthodox	75% withered normal CTC
Terpenoid (T)	4·10	5·45	2·15
Nonterpenoid (NT)	7·19	5·66	8·41
Total of T + NT	11·29	11·11	10·56
T/NT ratio	0·57	0·96	0·26

Seasonal changes

In Indian tea, there is a well defined season during May/June (second flush) when the flavour is outstanding. Figure 4 shows how the amount of terpenoids in the second flush teas while fat degradation products were higher in monsoon flushes. Figures 5–7 show the seasonal variations of carotenoids, chlorophylls and fatty acids. Significant variations of chlorophyll and carotenoids could be observed between second flush and monsoon flush though no such variations have been observed in the fatty acid compositions. Cambod clones are better producers of chlorophylls while
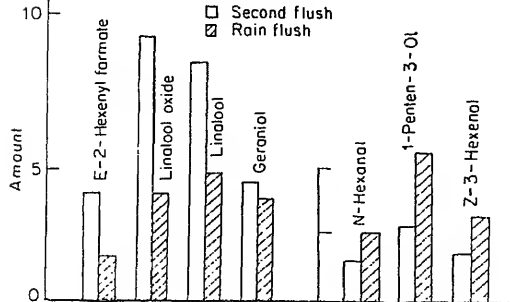
Figure 4. Difference in volatile flavour components of second and monsoon flushes.
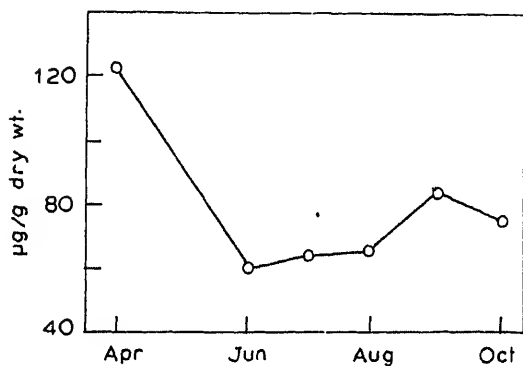
Figure 5. Seasonal variation of β-carotene.
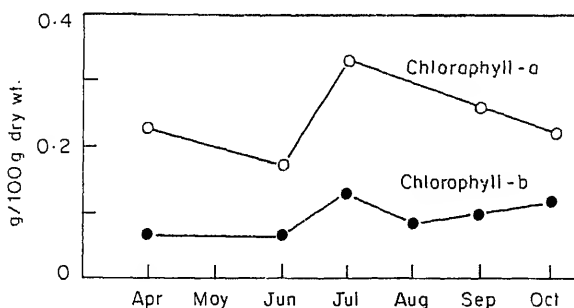
Figure 6. Seasonal changes of chlorophylls.

released during processing give rise to volatiles like alkanals, alkenols etc to impart sweet green note in made tea (Hatanaka and Harada, 1973).

Conclusion
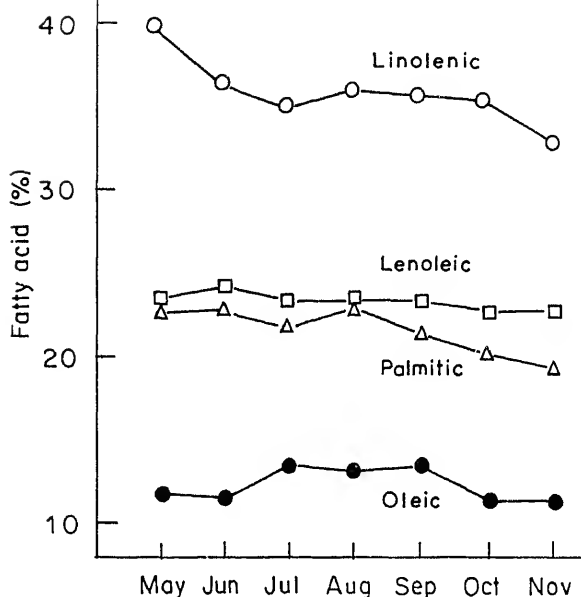
Figure 7. Seasonal changes of fatty acids.

quality clones may be carried out which would greatly reduce the potential difficulties associated with sensory methods.

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No. 117.

second author's name 'K. MISRA' should read as 'K. K. MISRA'.

No. 159.

Introduction line number 2 'serum prealbumin' should read as 'serum
min'.

$_{RK2}$ replicon function in the absence of *trfA* in *Azotobacter vinelandii*

S. SHANMUGASUNDARAM and P. M. MURALI

Department of Microbiology, School of Biological Sciences, Madurai Kamaraj University, Madurai 625 021, India

Abstract. The $oriV_{RK2}$ does not need the function of either $trfA^+$ or *trfA* operon for replication and maintenance of an $oriV_{RK2}$-containing plasmid in *Azotobacter vinelandii*.

Keywords. *trfA* deletion; $oriV_{RK2}$ replicon.

luction

tenance of a plasmid in a given bacterial host largely depends upon its ation and/or separation proficiency. Among the many, the IncP-1 plasmid has been the focus of study to determine the basis of IncP-1 plasmid enance in various Gram-negative bacteria. RK2, a 56·4 kilobase pair (kbp) ansmissible plasmid, has, in addition to genes concerned with replication, *kil* (*kilA*, *kilB1*, *kilB2* and *kilC*), which are potentially lethal to *Escherichia coli* cells, and *kor* genes (*korA*, *korB* and *korC*), which override the lethal action of l genes (Figurski *et al.*, 1982). *KorA* negatively controls *kilA* and *kilB1* but has itive effect on the expression of *korC*. *KorB* controls *kilB2* and *korC* regulates It is now clear that *kil* and *kor* functions are involved in the control of RK2 ation. It has been shown earlier that a 700-base-pair (bp) origin of replication and a *trans*-acting function, *trfA*, are essential for RK2 replication in *E. coli* er and Helinski, 1977; Figurski and Helinski, 1979; Thomas *et al.*, 1980). The peron which is 1·5 kbp long has, in addition to the *trfA* function, another one ated *kilD-kilB1* (Pohlman and Figurski, 1983; Smith and Thomas, 1983). fact *kilD* activity is known to destabilize the replication function of $oriV_{RK2}$ in bsence of the *kil* override function, designated *korD-korB1* (Pohlman and ski, 1983; Smith and Thomas, 1983). Interestingly it was also demonstrated in the absence of *korD-korB1* function, deletion of *kilD-kilB1* function izes the replication of $oriV_{RK2}$ in *E. coli*. This genotype was designated *trfA** nas, 1981). Therefore RK2 mini replicons containing *oriV* and *trfA** ΔkilD) were more stable in *E. coli* than those replicons which contain *oriV* and peron. However, these differences were not pronounced in *Azotobacter* sp. e in the present study the influence of a total deletion of the *trfA* region on the ation and maintenance of $oriV_{RK2}$ in *A. vinelandii* was investigated using an *in*

Table 1. Bacteria and plasmids used.

Strains of bacteria	Relevant genotype	Source/reference
E. coli HB101	F⁻, hsdS20 (r⁻B, m⁻B), recA13, ara-14, proA2, lacY1, galK2, rpsL20 (Smʳ) xyl-5, mtl-1 supE44, lambda	Laboratory collection (MKU, India).
A. vinelandii	Prototroph	Laboratory collection (Battelle, USA)
Plasmids		
pBR322	ampᴿ tetᴿ	Laboratory collection
pRK293	kanᴿ tetᴿ	Ditta et al. (1985)
pSBS-1	ampᴿ, kanᴿ, tetᴿ	Present study

Growth of bacteria

E. coli was grown in Luria Bertani (LB) broth and A. vinelandii in Burk's medium with and without combined nitrogen and at 25° and 30°C (Wilson and Knight, 1952). Competent cells of E. coli and A. vinelandii were prepared following Kushner (1978) and Glick et al. (1985) respectively. Whenever needed, tetracycline at $20 \, \mu g/ml$, ampicillin at $50 \, \mu g/ml$ and kanamycin at $20 \, \mu g/ml$ were used for E. coli. Ampicillin and kanamycin at concentrations of 15 and $5 \, \mu g/ml$ respectively were used for A. vinelandii.

DNA isolation

Plasmids pRK293 and pBR322 were extracted from E. coli as described by Maniatis et al. (1982) and purified by using cesium chloride density gradient containing ethidium bromide. The plasmids were linearized by treatment with restriction endonucleases EcoRI and SalI. The buffer and conditions used for restriction endonuclease treatment were as outlined by Bethesda Research Laboratories, USA.

Construction of recombinant replicon

Plasmids pRK293 and pBR322 were linearized and fragmented by treatment with EcoRI and SalI. Fragments derived from pRK293 were dephosphorylated using calf intestinal alkaline phosphatase (Machida and Ikeda, 1983). DNA fragments were ligated using T4 DNA ligase and were used to transform competent E. coli HB101 cells. Colonies grown on selective LB plates containing (i) kanamycin, (ii) ampicillin and kanamycin, and (iii) ampicillin, kanamycin and tetracycline were screened for the presence of plasmids.

Results

p. These were dephosphorylated using calf intestinal alkaline phosphatase. d pBR322 was digested with *Eco*RI and *Sal*I to two fragments of 3·7 kb and respectively. The 17·6 kb fragment of pRK293 contained the *trfA* region and kb fragment contained the *oriV*$_{RK2}$ and the gene encoding resistance for ycin. The 3·7 kb fragment of pBR322 contained *ori* of ColEl and the gene g resistance to ampicillin. The dephosphorylated pRK293 fragments were with the fragments of pBR322. The resultant recombinant replicons (figures 2) were used for further studies.
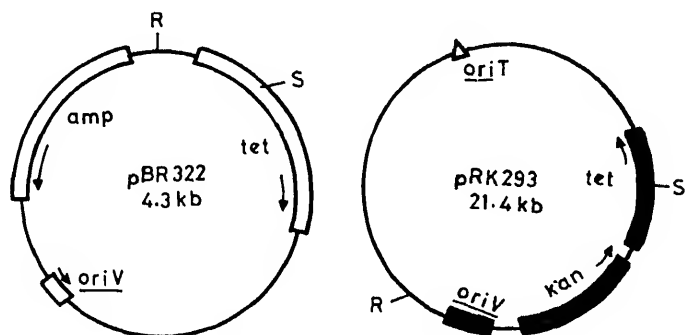
EcoRI, SalI digestion and ligation
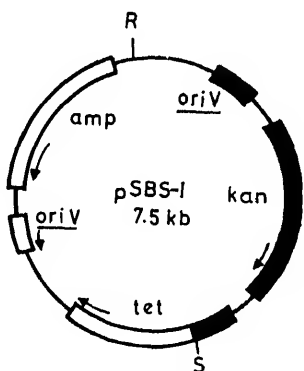
Figure 1. Construction of pSBS-1. *oriV*, Origin of replication; *oriT*, origin of transfer; kan, kanamycin resistance gene; tet, tetracycline resistance gene; amp, ampicillin resistance gene.

c transformation of E. coli

binant replicons constructed *in vitro* were used to transform competent cells. Transformants were screened by plating on selective plates containing amycin, (ii) kanamycin and ampicillin, and (iii) kanamycin, ampicillin and cline. In all 3 cases, 20 transformants per ng of DNA were obtained. The s that appeared on kanamycin plates, when replica-plated, were able to grow
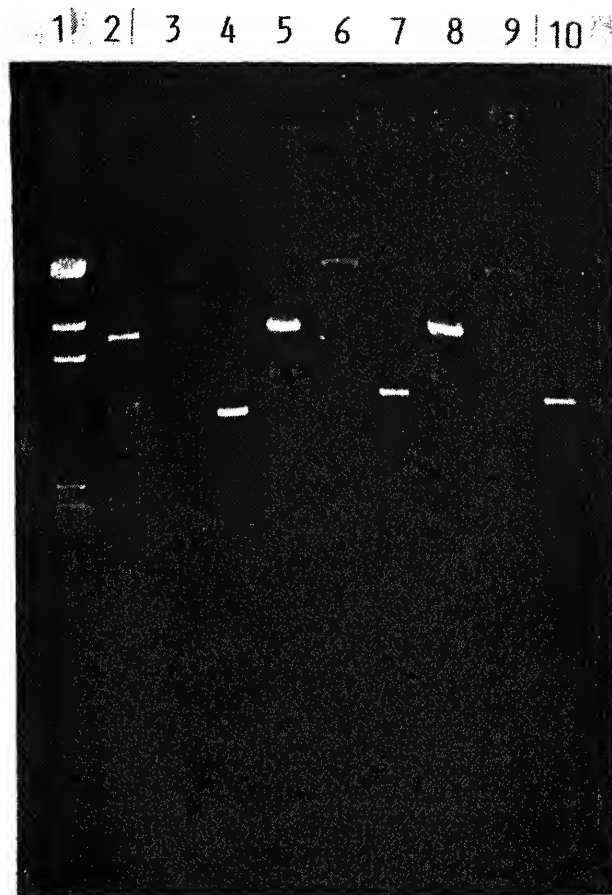
Figure 2. Restriction patterns of pSBS-1, pRK293 and pBR322. Lane 1: Lambda DNA digested with *Hind*III (markers). Lanes 2,3,4: pSBS-1, pRK293 and pBR322 digested with *Eco*RI and *Sal*I. Lanes 5,6,7: pSBS-1, pRK293 and pBR322 digested with *Eco*RI. Lanes 8,9,10: pSBS-1, pRK293 and pBR322 digested with *Sal*I.

Genetic transformation of A. vinelandii

The hybrid replicon constructed was named pSBS-1. It contains *ori* of ColEl, *oriV* of RK2 and genes coding for resistance to ampicillin, tetracycline and kana-mycin. Plasmid pSBS-1 purified over cesium chloride/ethidium bromide buoyant density gradient was used to transform competent *A. vinelandii* cells. The

growth conditions are presented in table 2. There was no difference in the
of colonies when aliquots were plated at periodical intervals on plates
ng antibiotics and plates lacking antibiotics. A few colonies were routinely
for the presence of plasmids and were found to contain them. So far none
RK2-derived mini-replicons have been shown to follow a chromosomal
ion and excision cycle during its replication in any Gram-negative
m. The presence of pSBS-1 was always noticed in all mini-preps made from
ndii transformants. This demonstrates that pSBS-1 could be maintained in
ndii in the absence of any selective pressure for many generations (>20).

Table 2. Generation time (GT) of *A. vinelandii* transformants
under various growth conditions.

Medium	Temp. (°C)	Aeration condition	GT (h)	Lag period (h)
N−[a]	25	still[b]	9·6	40
N−	25	shake[c]	4·8	20
N+	25	still	8·0	40
N+	25	shake	4·0	20
N−	30	still	9·6	24
N−	30	shake	3·8	<2
N+	30	still	8·0	24
N+	30	shake	3·2	<2

[a]N in N+ medium was $NH_4 = 0.036$ M given as NH_4 acetate.
[b]Cells grown as still culture. [c]Cells grown in a shaker
(100 rpm).

ion

orid replicon pSBS-1 was constructed after totally deleting the *trfA* operon
functions of RK2. The replication of pSBS-1 in *E. coli* is due to the
e of the *ori* of ColEl. A construction with only $oriV_{RK2}$ was not obtained in
sent study as all transformants selected on kanamycin plates were resistant
cillin and tetracycline. Ligation of the 3·8 kbp fragment of pRK293 and the
fragment of pBR322 would have yielded a replicon with $oriV_{RK2}$, conferring
ce to kanamycin only.
results are also in agreement with earlier observations where *trfA* function
wn to be necessary in addition to $oriV_{RK2}$ for replication and maintenance
in *E. coli* (Schmidhauser and Helinski, 1985). However, it is evident from
sent study that *trfA* function does not seem to be important for replication
intenance in *A. vinelandii* since pSBS-1 replicates and maintains itself well in
ndii in the absence of *trfA* gene(s). The recent observation of Smith and
s (1987) confirms our observation. The other IncP plasmid pHH502-1, which
equence homologous to $oriV_{RK2}$, does not have a sequence homologous to
mith and Thomas 1987) the essential replication gene of RK2 in *E. coli* and

Acknowledgement

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ic fluorescence polarization studies on lipid mobilities in olipid vesicles in the presence of calcium mediators

PURNIMA KAUL and V. KOTHEKAR

Department of Biophysics, All India Institute of Medical Sciences, New Delhi 110 029, India

Abstract. The influence of Ca^{2+} mediators (nifedipine, verapamil and prostaglandin $F_{2\alpha}$) on fluorescence polarization of 1-anilino-8-napthalene-sulphonate in dipalmitoyl phosphatidylcholine and dimyristoyl phosphatidylcholine liposomes was studied at various temperatures to understand the dynamic behaviour of membrane lipids. We also studied the effect of change in calcium concentration on the fluorescence polarization of the dye in the liposomes. Our results show increase in polarization (indicative of stiffening of the membrane) in the presence of Ca^{2+} ions. In the case of dimyristoyl phosphatidylcholine liposomes, all 3 drugs caused decrease in fluorescence polarization (increase in fluidity of the membrane) with or without Ca^{2+} ions in the medium. Contrary to this, in the case of dipalmitoyl phosphatidylcholine liposomes, the fluidization effect is observed for all the 3 drugs in the absence of Ca^{2+} ions; in the presence of Ca^{2+} ions stiffening is observed upon addition of nifedipine and verapamil which are antagonists, and fluidization is observed upon addition of prostaglandin $F_{2\alpha}$. The role of drug-induced fluidity changes in membranes in therapy planning is discussed in the paper.

Keywords. Fluorescence. polarization; lipid mobilities; phospholipid vesicles; calcium mediators.

tion

ally diverse groups of organic compounds are known to be effective as mediators (Bolton, 1979; Swamy and Triggle, 1986). This suggests plurality ode of action. The molecular architectures of the Ca^{2+} mediators have no ling common feature. A wide spectrum of chemical structures usually means f stereo-specificity in the drug's action and is indicative of the interaction of s with membrane lipids rather than with specific membrane proteins (Shi n, 1986). It has been reported that the depression of Ca^{2+} uptake caused by inhibitors may result from general perturbation of membrane lipids st *et al.*, 1980).

nain aim of our present study is to understand the physico-chemical basis he differential action of Ca^{2+} mediators. For this purpose we have chosen 3 iz., nifedipine, verapamil and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (figure 1), the first ng Ca^{2+} inhibitors and the last one a calcium activator. Earlier, we have (Purnima and Kothekar, 1988a, b) the conformational flexibility and ar electrostatic potential distribution of these drugs, and the binding s of their interaction with liposomes using 1-anilino-8-naphthalene sulphonate
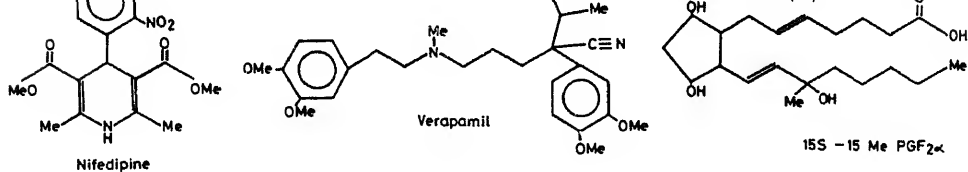
Figure 1. Structures of (A) nifedipine, (B) verapamil and (C) PGF$_{2\alpha}$.

of these mediators. We have also considered the effect of calcium ion on fluidization of the membrane. This, we believe, is one of the best approaches available for studying the mechanisms of drug-mediated membrane processes, because lipid fluidity determines the lateral and rotational freedom of the mobile membrane proteins and can modulate the degree of exposure of membrane proteins (Shinitzky and Henkart, 1979).

Lipid fluidity was studied using the fluorescence polarization technique. As in our earlier paper, we have used the fluorescent dye ANS. Binding of this amphiphilic dye to phospholipid assemblies was reported to be a sensitive indicator of lipid assembly (Slavik, 1982). On the basis of X-ray diffraction (Lesslauer et al., 1972) and nuclear magnetic resonance studies (Podo and Blasie, 1977) bound ANS is known to be located at the phospholipid polar heads. The binding is mainly due to hydrophobic forces but is also determined by the electrostatic interactions arising from the surface charges (Maclaughlin and Harary, 1976; Teissie, 1979). The interaction of these drugs with membranes was studied using dipalmitoyl phosphatidylcholine (DPPC) and dimyristoyl phosphatidylcholine (DMPC) liposomes. The reason for choosing lecithin (phosphatidylcholine) for this study is, that it is a major component of many biological membranes. Moreover, DPPC and DMPC have well-defined chemical structures. DPPC has transition temperature well above room temperature. We chose two different fatty acid side-chains because fatty acid chain lengths markedly influence the activity of membrane transport processes (Overath et al., 1970; Wilson et al., 1970; Esfahani et al., 1971).

Materials and methods

Chemicals

DPPC, DMPC, verapamil, nifedipine, Tris buffer and ANS were from Sigma Chemical Co., St Louis, Missouri, USA. Prostaglandin 15S-15-Me PGF$_{2\alpha}$ was purchased from Upjohn Company, England. All other routine chemicals were of Analar grade from British Drug House, Bombay.

Phospholipid vesicles

Unilamellar vesicles were prepared by sonication (Bangham et al., 1965). This

olipid in chloroform is dried in a glass tube under a stream of nitrogen and
evaporated. This results in the formation of a thin lipid film on the wall of
e. Final traces of solvent are removed after leaving the tube *in vacuo* for 4–
e desired amount of Tris buffer (0·01 M, pH 7·4) is added to produce a lipid
ion. It is vortexed for 10 min at 35°–40°C. The lipid suspension is then
ed to clarity in a probe-type sonifier cell disruptor such as the Branson B-30.
sulting vesicles are viewed in a Philips EM 301 transmission electron
ope after negative staining. They are of fairly uniform size (200–300 Å).

cence polarization spectroscopy

fluorescence polarization measurements were made with a Union Giken
ence spectrophotometer, fitted with temperature control attachment and
ic stirrer. The polarization P was calculated as:

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}},$$

$G = I_{HV}/I_{HH}$ is the grating correction factor. Subscripts V and H refer to
and horizontal orientation of polarizer or analyser. The static polarization
ated to order parameter $S = \langle 3\cos^2\theta - 1 \rangle$ by a relation

$$P = \frac{3\cos^2\theta - 1}{\cos\theta + 3}.$$

oaches zero when molecules tumble very fast. Contrary to this, increase in P
ative of increase in θ and order parameter. Thus fluorescence polarization
ement gives a direct idea of membrane fluidity.
rescence polarization monitors phospholipid phase transition as a sharp
In polarization in the region of T_c (gel-liquid-crystalline transition
ature). The decrease in polarization corresponds to the marked increase in
ational freedom of the probe upon melting of phospholipid acyl chains
n and Lussan, 1973; Jacobson and Papahadjopoulos, 1975).
l experiments the concentrations of ANS and lipids were held fixed at 10^{-5}
8×10^{-5} M respectively. The effect of the drugs (nifedipine, verapamil and
) on fluorescence polarization was measured at 4° intervals between 22° and
or DPPC liposomes and 10° and 34°C for DMPC liposomes using a fixed
oncentration of 4×10^{-5} M (figure 2).
transition temperature was calculated by plotting gradient $\Delta P/\Delta T$ as a
n of temperature T. It is given in table 1. Effect of Ca^{2+} ion on ANS
cence was studied in the absence and presence of the drugs. Incorporation of
without' any drug leads to increase in observed fluorescence (figure 3).
scence polarization of ANS shows increase upon addition of Ca^{2+} ion to
. However, its value remains constant between Ca^{2+} concentrations of 0·57
6 mM. It shows a sharp rise when Ca^{2+} goes above 4 mM. Hence we chose
M Ca^{2+} concentration for studying the effect of Ca^{2+} ion on fluorescence
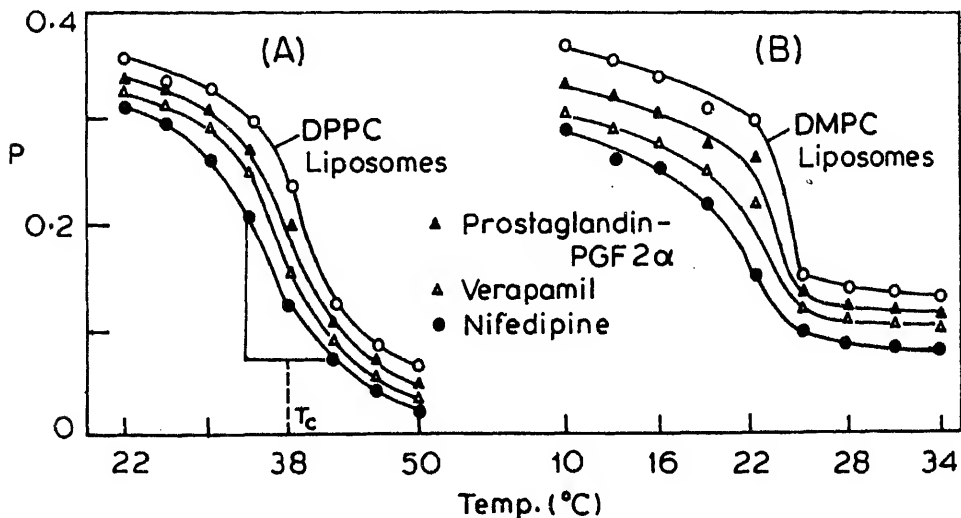
Figure 2. Change with temperature of fluorescence polarization P in (A) DPPC liposomes and (B) DMPC liposomes in the presence of nifedipine, verapamil and $PGF_{2\alpha}$. T_c is calculated by plotting $\Delta P/\Delta T$ vs T and taking the maximum.

Table 1. Change in gel-liquid-crystalline transition temperature T_c of DPPC and DMPC liposomes due to nifedipine, verapamil and $PGF_{2\alpha}$ without Ca^{2+} and with 0.57 mM Ca^{2+}.

Lipid	Drug	T_c (without Ca^{2+}) (°C)	T_c (with Ca^{2+}) (°C)
DPPC	—	41	—
	Nifedipine	37·0	48·0
	Verapamil	37·0	44·0
	$PGF_{2\alpha}$	39·0	37·0
DMPC	—	23·5	—
	Nifedipine	22·0	21·5
	Verapamil	22·5	22·0
	$PGF_{2\alpha}$	22·5	22·0

Concentration of ANS 10^{-5} M, lipid 1.08×10^{-5} M and drugs 4×10^{-5} M.

Results and discussion

Effects of Ca^{2+} mediators on membrane fluidity

Figure 2 shows the effect of nifedipine, verapamil and $PGF_{2\alpha}$ in DPPC and DMPC liposomes at various temperatures. It has been observed that the value of P
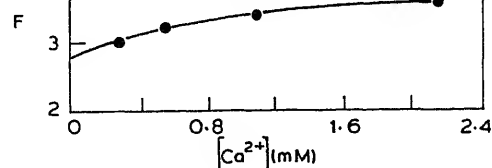
Figure 3. Fluorescence F of ANS in DPPC liposomes as a function of Ca^{2+} ion concentration (mM).
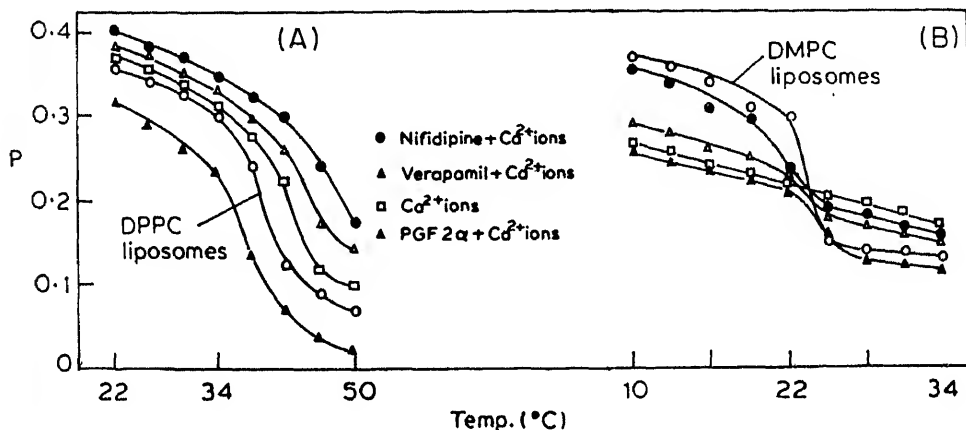
Figure 4. Change with temperature of the fluorescence polarization P in (A) DPPC liposomes and (B) DMPC liposomes in the presence of nifedipine, verapamil and $PGF_{2\alpha}$ and 0.54 mM of Ca^{2+}.

addition of nifedipine and verapamil to DPPC liposomes, and by 2°C on addition of $PGF_{2\alpha}$ (table 1). In the case of DMPC the reduction in the transition temperature is 1·5°C on addition of nifedipine and 1°C on addition of verapamil and $PGF_{2\alpha}$.

Thus all the 3 drugs can interact with the lipid portion of the membrane and fluidize it. Similar observations had been reported by Erdreich and Rahamimoff (1984) for verapamil using sarcolemmal vesicles. The authors observed that the inhibition of Ca^{2+} uptake could be reversed by addition of phosphatidylcholine. Reduction in the transition temperature of DMPC liposomes due to verapamil was noted by Shi and Tien (1986) using electron spin resonance spectroscopy. Thayer et al. (1985) reported that verapamil and D-600 impaired the binding function of their receptors through perturbation of membrane fluidity. Rearrangement of phospholipids in erythrocytes in the presence of a small amount of PGE_1 was noted by Manevich et al. (1985). Kury and McConnel (1975) attributed this to increase in lipid fluidity. However, there had been to comparative study of fluidity changes in the same membrane preparation caused by Ca^{2+} mediators belonging to different chemical groups.

We observed that nifedipine brought about the maximum change in fluorescence

found to be well correlated with the binding constants ($2\cdot4 \times 10^4$, $1\cdot65 \times 10^4$ and $1\cdot4 \times 10^4$) for the interaction nifedipine, verapamil and $PGF_{2\alpha}$ with DPPC liposomes measured earlier (Purnima and Kothekar, 1988a). It had been suggested by Shi and Tien (1986) that fluidity effect of different drugs on membrane lipids depend on their lipophilicity. In the theory proposed by Lee (1976) for sodium channels, fluidity of the lipid moiety leads to closing of the channels. Whether a similar model can be used to explain the pharmacological function of Ca^{2+} mediators is not known. The greater effect of nifedipine is indicative of the fact that the theory may hold true for calcium mediators also. In our study $PGF_{2\alpha}$ shows the least effect and it is an agonist. Thus, effect on fluidity of lipid membranes is a useful indicator of the differential activity of Ca^{2+} mediators.

However, we cannot overlook the fact that the physiological mechanism for Ca^{2+} transport is more complex and involves many parameters, such as state of membrane polarization, ion fluxes, extracellular calcium levels, etc. (Akiyama and Gish, 1979; Herbette *et al.*, 1983; Reddy *et al.*, 1984; Kothekar *et al.*, 1985). We have studied the influence of calcium ion on lipid fluidity of DPPC and DMPC vesicles in the presence of 3 drugs to probe further into the interrelationship between various factors controlling Ca^{2+} transport.

Effect of Ca^{2+} mediators on membrane fluidity in the presence of Ca^{2+} ions

Figure 4a shows that addition of Ca^{2+} ion (0·54 mM) to DPPC liposomes increases fluorescence polarization, which means that it causes stiffening of the membrane. This is due to increase in the lipid order parameter S, which shifts the transition temperature to the higher side (Trauble and Eibl, 1974; Eibl and Blume, 1979). Decrease in probe mobility was also noticed by Ashley and Brammer (1984) upon addition of $CaCl_2$ to synaptosomal lipid extracts.

Addition of nifedipine and verapamil lead to further increase of P and increase in the transition temperature by 7° and 3°C respectively. Contrary to this, addition of $PGF_{2\alpha}$ leads to decrease of P and reduction of T_c by 2°C which means that there is fluidization of the membrane like in the case of action of anaesthetics observed by Papahadjopoulos *et al.* (1975). This result shows the complexity of the phenomenon and the need for a detailed study of the interaction between channel-forming proteins and lipids in the presence of drugs and other molecules.

In the case of DMPC liposomes (figure 4b), the presence of Ca^{2+} leads to increase in P and disappearance of gel-liquid-crystalline transition. Addition. of nifedipine, verapamil and $PGF_{2\alpha}$ leads to a complicated behaviour. Transition temperature decreases by 2°C upon addition of nifedipine. Verapamil and $PGF_{2\alpha}$ cause a reduction in T_c by 1·5°C. At any temperature $PGF_{2\alpha}$ brings about the maximum fluidization.

l intra- and intermolecular interactions suffice to produce the observed
hese observations may have relevance in drug therapy, since changes in
mposition of cell membranes can now be achieved by dietic and other
The latter can provide the key tools for reversible modulation of membrane
    Many studies have attempted to relate certain disease conditions to
tion of biomembrane fluidity.

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t of light on nucleotide modifications in the transfer RNA of
ıber cotyledons

M. PUTTA RAJU and C. JAYABASKARAN

Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India

Abstract. The effect of light on nucleotide modifications in the tRNA of cucumber (*Cucumis sativus* L. var. Guntur) cotyledons was studied by chromatographic, electrophoretic and immunological methods. The tRNA from light-grown tissue showed the absence of 2-methylguanosine and a decrease in the relative proportions of ribothymidine and cytokinin-active ribonucleosides when compared to those produced from dark-grown tissue. On the other hand, a significant amount of one species of 2′-O-methyldinucleotide was observed in the tRNA of light-grown tissue which was not detected in the dark-grown tissue. Also, tRNA from light-grown tissue had higher levels of another species of 2′-O-methyldinucleotide. The results showed no difference in the amounts of other modified nucleosides in tRNA between tissues grown under the two conditions. 2′-O-Methyl-1-methyladenosine, a nucleotide modified both in the base and the ribose, apparently specific to plant tRNAs, has been found to be present in the RNA of both light- and dark-grown tissues. These results on the variation in modified nucleotides suggest that light has some role in nucleotide modification and, consequently, in cellular functions.

Keywords. tRNA; light; modified nucleotides; *Cucumis sativus*.

ıction

er RNA contains a large number of modified nucleosides. More than 50 ıt types of modified nucleosides have been isolated and characterized in (Nishimura, 1979; Bjork *et al.*, 1987). It is increasingly recognized that ed nucleotides in tRNA play important roles in protein synthesis as well as ulation, but the mechanism of their action is not yet clear. There is a great f experimental evidence suggesting the involvement of individual modified sides in functions such as codon–anticodon interactions, preferential codon ition, interaction with ribosomes and regulation of gene expression nura, 1979; Bjork *et al.*, 1987).

opears that organisms regulate their metabolism in response to environmental ices by modifying the nucleosides in their tRNA, thereby modulating the it functions of the molecule (Ajitkumar and Cherayil, 1988). Buck and ıs (1981) have observed that *Escherichia coli* tRNA^{Phe}, tRNA^{Trp} and tRNA^{Tyr} usually have ms^{2}i^{6}A contain i^{6}A under iron-restricted growth conditions.

These tRNAs were found to be translationally less efficient and apparently function as regulatory elements in the expression of certain operons of the aromatic amino acid biosynthetic pathway. It has been shown that tRNA from *Azotobacter vinelandii* grown in the presence of ammonium chloride lacks T while that from cells grown in the absence of the ammonium salt contains this modified nucleoside (Ajitkumar and Cherayil, 1982). The observation that loss of a modified nucleoside from tRNA can affect the regulatory processes of the cell (Singer *et al.*, 1972; Yanofsky and Söll, 1977) has given support to the concept that changes in levels of modified nucleosides during growth and differentiation have physiological importance.

Information on the modification of plant tRNA is scanty apart from information on the identification of cytokinin-active ribonucleosides. The present investigation was therefore intended to make a systematic analysis of modified nucleosides from a plant tRNA. The cotyledons excised from seedlings of cucumber, which become green and photosynthetic when exposed to light in the presence of water, provide a useful system for the study of modified nucleotides in tRNA. In the present work, changes in modified nucleotide levels were studied in light- and dark-grown cotyledons by comparing the nucleotide patterns of tRNA samples by chromatographic, electrophoretic and immunological methods.

Materials and methods

Chemicals

i^6A, io^6A (predominantly the *trans* isomer, containing approximately 8% of the *cis* isomer), bovine serum albumin (BSA), DEAE-Sephadex A-25, bovine spleen phosphodiesterase, nuclease P1 and ribonuclease T_2 were obtained from Sigma Chemical Co., St Louis, Missouri, USA. Bacterial alkaline phosphatase and snake venom phosphodiesterase were from Worthington Biochemical Corporation, USA. Plastic-backed cellulose thin-layer plates were from Macherey-Nagel, FRG. Cellulose acetate membrane strips and DEAE-cellulose paper were from Schleicher and Schuell, Keene, New Hampshire, USA. Nitrocellulose filters ($0.45\ \mu m$; MDI filters) were from Microdevices, Ambala. Carrier-free [^{32}P]orthophosphoric acid was obtained from Bhabha Atomic Research Centre, Bombay. The seeds of *Cucumis sativus* L. var. Guntur were provided by Karnataka Seed Corporation, Bangalore. All other chemicals and reagents used were of analytical grade available commercially.

Plant materials

Cucumber (*Cucumis sativus* L. var. Guntur) seeds were surface-sterilized with 0.1% mercuric chloride solution, and then rinsed several times in distilled water. Seeds were allowed to germinate on moist Whatman No. 1 paper discs in sterilized Petri

wetted with sterile water. The cotyledons were washed with distilled water and blotted before using them for tRNA extraction.

Isolation of tRNA

Total tRNA was prepared from cucumber cotyledons by the phenol–sodium dodecyl sulphate (SDS) method (Jayabaskaran and Jacob, 1982) followed by DEAE-cellulose chromatography. Contaminating polysaccharides were removed by extraction of RNA into 2-methoxyethanol and precipitation by cetyltrimethylammonium bromide (Bellamy and Ralpha, 1968) followed by electroelution of tRNA from an 8% polyacrylamide gel.

Enzymatic digestions

$[^{32}P]$tRNA was hydrolysed to nucleotides by digestion with RNase T_2 (5 U of enzyme for 25 A_{260} units of tRNA) in 50 mM ammonium acetate buffer (pH 4·5) containing 1 mM EDTA at 37°C for 16 h. Unlabelled tRNA was digested to nucleosides by incubating approximately 500 μg of tRNA in 0·5 ml of 0·2 M Tris-HCl buffer (pH 8·5) with 50 μg of snake venom phosphodiesterase and 20 μg of alkaline phosphatase for 16 h at 37°C. Digestion of ^{32}P-labelled RNase T_2-resistant dinucleotides with 0·05 U of spleen phosphodiesterase and 20 μg of nuclease P1 were carried out at 37°C in 50 mM ammonium acetate pH 6 and pH 5·3 respectively. ^{32}P-Labelled RNase T_2-resistant dinucleotides were dephosphorylated by incubating about 3 U of bacterial alkaline phosphatase in 50 mM Tris-HCl buffer (pH 8·8) for 3 h at 37°C.

High voltage paper electrophoresis and chromatography

High voltage electrophoresis on Whatman No. 3 paper and cellulose acetate membrane strips was carried out in pyridine–acetate–EDTA buffer, pH 3·5, at 50–60 V/cm using xylene cyanol and acid fuchsin as dye markers (Smith, 1967). Two-dimensional electrophoresis using cellulose acetate and DEAE-cellulose paper was performed according to Cory and Adams (1975). The RNase T_2 digest of the tRNA was resolved into mononucleotides and dinucleotides by chromatography on a DEAE-Sephadex A-25 column (Watanabe et al., 1979). The column (23 × 0·3 cm) was developed using a linear gradient of 200 ml each of 0·12 M and 0·4 M NaCl in 20 mM Tris-HCl, pH 7·4, containing 7 M urea. Two-dimensional thin-layer chromatography (TLC) was performed according to the method of Nishimura (1979) in the following solvent systems: (i) isobutyric acid: 0·5 M NH_3 5:3 (v/v) and (ii) isopropanol: HCl: H_2O, 70:15:15 (v/v/v). Sephadex LH-20 column chromatography was used to separate and characterize cytokinins (Jayabaskaran and Jacob, 1982).

Radioimmunoassay

conjugated to BSA and characterized as already described (Humayun and Jacob, 1974; Milstone *et al.*, 1978; Hofman *et al.*, 1986). The radioimmunoassays for i^6A and io^6A were performed as described previously (Jayabaskaran and Jacob, 1982). Briefly, the reaction mixture in a total volume of 0·4 ml Tris-buffered saline (TBS), contained the respective anti-serum and tritiated cytokinin with or without inhibitor. After incubation at 37°C for 10 min, the contents were filtered on prewetted nitrocellulose filter, washed with TBS, dried and counted for radioactivity in 0·5% PPO in toluene in a liquid scintillation counter. i^6A and io^6Ade were randomly labelled with tritium by exposure to tritium gas by Bhabha Atomic Research Centre, Bombay. The specific activities of the $[^3H]\text{-}i^6A$ and $[^3H]\text{-}io^6Ade$ prepared by this technique were 10,000 cpm/mol and 20,000 cpm/pmol respectively (uncorrected for counting efficiency and other sources of error). These compounds were characterized by chromatography on Sephadex LH-20 column and by UV absorption spectrophotometry.

Results

Nucleotide analysis of $[^{32}P]tRNA$

^{32}P-Labelled total tRNAs isolated from light- and dark-grown cotyledons of *C. sativus* were digested with RNase T_2 and the products of the two samples of tRNA were analysed by two-dimensional TLC. The presence of modified nucleotides such as m^1Ap, m^7Gp, m^5Cp, m^6Ap, m^2Ap, Tp, Dp and i^6A derivatives and dinucleotides such as CmpCp, AmpAp, GmpGp, CmpGp/GmpCp and GmpAp/AmpGp were detected. In addition to the four major nucleotides, pGp and pUp were observed (figure 1). RNase T_2 produces, in addition to the mononucleotides, dinucleotides from sites of which the 2'-oxygen of a nucleotide is methylated. The chromatogram of dark-grown tissue showed a spot in the region corresponding to m^2Gp but no trace of this nucleotide was detected in the tRNA isolated from light-grown tissue. Each area containing radioactivity was cut out, and was quantitated. The data are given in table 1. The Tp content of tRNA in light-grown tissue was about one half of that in dark-grown tissue: While dark-grown tissue had 0·71 mol% of Tp, light-grown tissue had only 0·32 mol%. On the other hand, in the dark-grown tissue, the GmpAp/AmpGp content was found to be low, only one half compared to that of tRNA from light-grown tissue. The results were reproducible and the data presented are the averages of 4 experiments. As can be seen in figure 1a and table 1, no trace of CmpAp/AmpCp was detected in the tRNA of dark-grown tissue.

The $[^{32}P]tRNA$ digest was subjected to paper electrophoresis on Whatman No. 3 paper at pH 3·5. The autoradiogram showed the presence of many species, numbered 1–10, in addition to the spots due to the 4 major nucleotides in both the samples of tRNA (figure 2). Spots 2 and 10 were further analysed and shown to be m^5Cp and 3',5'-guanosine diphosphate (pGp), respectively (data not shown). In figure 2, the intensity of spot 10 in lane b is much lower than that of the corres-
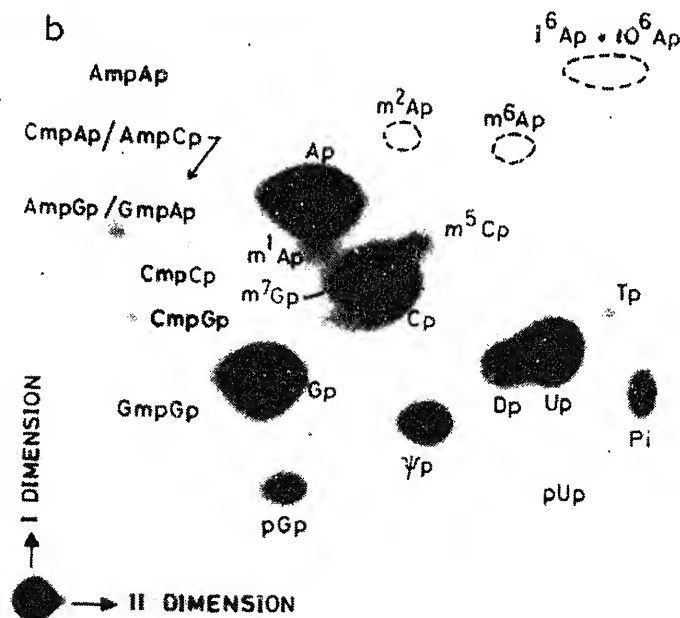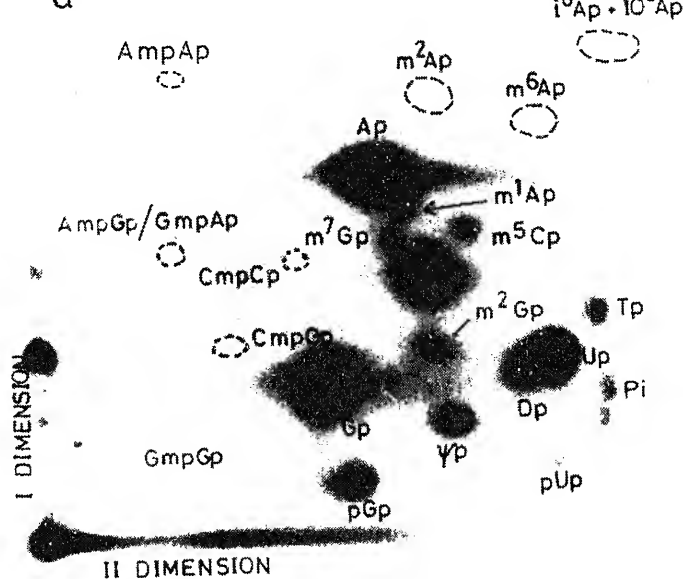
Figure 1. Autoradiogram of two-dimensional TLC of RNase T_2 digest of tRNA samples. RNase T_2 digest of $[^{32}P]$tRNA samples were subjected to two-dimensional TLC on cellulose plates. The dotted circles show the positions of nucleotides present in too low an amount to be reproduced photographically. Samples from (a) dark-grown cotyledons and

Table 1. Relative proportions of nucleotides in tRNA from light- and dark-grown cucumber cotyledons.

	mol%*					
	By two-dimensional thin-layer chromatography (figure 1)		By high voltage electrophoresis on Whatman paper (figure 2)		By two-dimensional electrophoresis	
Nucleotide	Dark-grown	Light-grown	Dark-grown	Light-grown	Dark-grown	Light-grown
Cp	24·21	24·54	25·13	24·64	24·75	26·29
Ap	21·73	21·38	20·58	21·79	21·58	21·64
Gp	26·00	27·24	27·35	28·17	27·68	27·40
Up	18·23	18·04	22·66	21·60	21·93	20·53
m^1Ap	0·25	0·17	0·21**	0·15**	—	—
m^7Gp	0·33	0·23	0·30**	0·22**	—	—
m^5Cp	0·45	0·44	0·48	0·42	0·45	0·50
Tp	0·71	0·32	—	—	—	—
Dp	3·15	3·28	—	—	—	—
Ψp	1·45	1·58	—	—	—	—
m^6Ap	+	+	—	—	—	—
m^2Ap	+	+	—	—	—	—
Cytokinins	+	+	—	—	—	—
m^2Gp	0·90	0·00	—	—	—	—
pGp	1·15	1·20	1·12	1·19	1·27	1·20
pUp	0·15	0·19	0·17	0·20	—	—
CmpCp	0·08	0·08	0·10	0·11	0·08	0·07
AmpAp	0·04	0·03	0·04	0·05	0·04	0·04
m^1AmpUp	—	—	0·04	0·05	—	—
GmpGp	0·15	0·11	—	—	0·14	0·10
UmpUp	—	—	0·06	0·05	0·06	0·05
CmpGp/GmpCp	0·04	0·05	0·05	0·06	0·08	0·06
UmpGp/GmpUp	—	—	0·21	0·23	0·23	0·25
CmpAp/AmpCp	0·00	0·08	—	—	0·00	0·09
CmpUp/UmpCp	—	—	—	—	0·08	0·09
GmpAp/AmpGp	0·03	0·05	—	—	0·03	0·07
AmpUp/UmpAp	—	—	—	—	0·06	0·07

*Mol% was obtained by cutting the corresponding spot from paper or thin layer and determining the radioactivity. The values are percentages of total radioactivity found in all spots on the electrophorogram or chromatogram.

**By high voltage electrophoresis on Whatman No. 3 paper (figure 2) $m^1Ap + m^7Gp$ are not resolved; upon elution and re-electrophoresis on cellulose acetate, resolved into m^1Ap and m^7Gp.

⁺Indicates that the nucleotide was present in the autoradiogram but its molar yield was not determined.

phoretic mobility, spot 1 was presumed to contain m^1Ap or m^7Gp or both. These base-methylated nucleotides occur frequently in eukaryotic tRNA and, because of an additional positive charge on the base, are known to have low anodic mobilities at pH 3·5. Analysis of spot 1 by electrophoresis on cellulose acetate (Silberklang *et al.*, 1979) and modification by treatment with alkali (Brown *et al.*, 1978) established that it contained both m^7Gp and m^1Ap. Spots 3–9 were identified as RNase T₂-
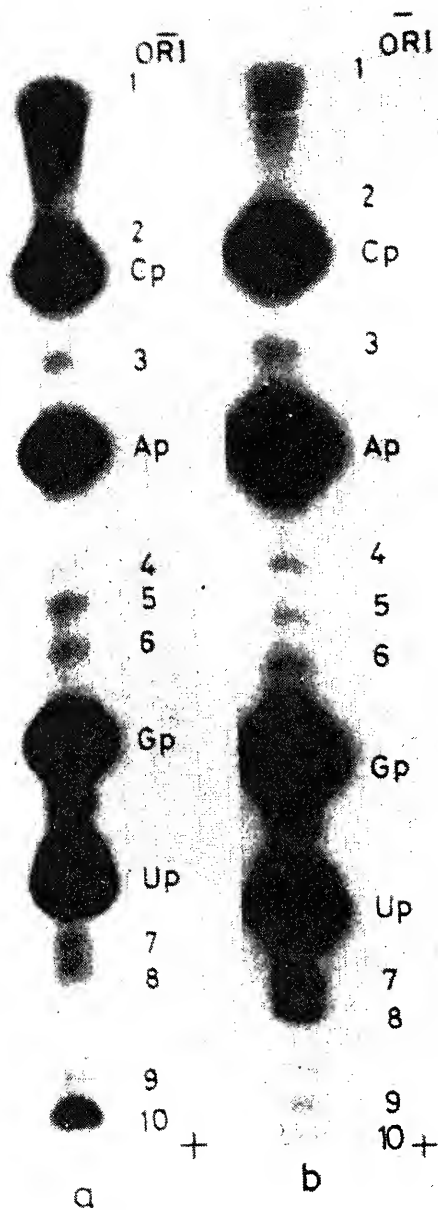
Figure 2. Autoradiogram of high voltage electrophoretic separation of nucleotides. RNase T_2 digests of $[^{32}P]$tRNA samples were subjected to electrophoresis at pH 3·5 at 60 V/cm on Whatman No. 3 paper. Samples from (a) dark-grown cotyledons and (b) light- grown cotyledons.

analysed by high voltage paper electrophoresis from the two conditions of growth are similar.

RNase T_2-resistant dinucleotides can be separated from the bulk of the mono-nucleotides by chromatography on DEAE-Sephadex A-25 in the presence of urea (Watanabe *et al.*, 1979). The chromatographic profiles of an RNase T_2 digest of light- and dark-grown cotyledon tRNA are shown in figure 3. Of the total radio-
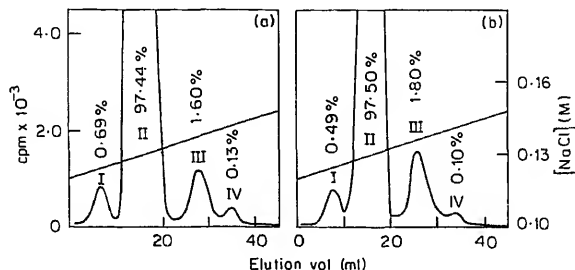
Figure 3. Elution profiles of the RNase T_2 digest of $[^{32}P]$tRNA samples from a DEAE-Sephadex A-25 column. The column (23 × 0·3 cm) was developed in the presence of 7 M urea with a linear gradient of 200 ml each of 0·12 M NaCl and 0·4 M NaCl in 20 mM Tris-HCl, pH 7·4. Peaks 1, 2 and 3 represent cyclic mononucleotides, 3'-mononucleotides and dinucleotides respectively. Peak 4 may contain the small fraction of trinucleotides arising out of two successive 2'-O-methyl group. The relative proportions of the peaks are indicated. (a), Dark-grown cotyledons; (b), Light-grown cotyledons.

activity, 1·8 and 1·6% eluted in the light- and dark-grown tissues, respectively, as the dinucleotide peak. Since the dinucleotide contains two phosphate groups, the mol% will be 0·9 and 0·8 respectively (table 1). Two-dimensional electrophoresis gives better resolution of dinucleotides compared to the above methods. The relative proportions of dinucleotides in the light- and dark-grown tissues were therefore verified by subjecting the RNase T_2 digest to two-dimensional electro-phoresis. The autoradiogram showed the presence of many dinucleotides (data not shown). The identities of the dinucleotides were derived by comparison with the pattern obtained by Cory and Adams (1975). Each of the spots was cut out and the radioactivity determined. The relative proportions of the dinucleotides analysed by the above methods are given in table 1.

Quantification of cytokinins

Unfortunately, cytokinins are not amenable to analysis by the above biochemical techniques because they are present in small amounts and are partially degraded during the analysis. These compounds migrate together to the upper right-hand corner in two-dimensional TLC (figure 1). Since we had made antibodies to i^6A and io^6A, we decided to employ antibodies to quantify i^6A and io^6A in tRNA samples.

kinins in the tRNA of light- and dark-grown tissues, the tRNAs were hydrolysed to nucleosides using snake venom phosphodiesterase and alkaline phosphatase and the nucleosides subsequently fractionated on Sephadex LH-20 column. Fractions containing i^6A and io^6A were pooled, dried, dissolved in TBS and quantitated by radioimmunoassays using the appropriate standard curves. The values are given in table 2. tRNA from dark-grown tissue has more of i^6A and io^6A, approximately 30 and 23% more, respectively, compared to tRNA from light-grown tissue.

Table 2. Levels of i^6A and io^6A in tRNA of light- and dark-grown cucumber cotyledons.

	Amount of cytokinins	
Sample	i^6A (pmol)	io^6A (pmol)
Light-grown	250	920
Dark-grown	356	1185

About 100 A_{260} units of total tRNA samples from light- and dark-grown cucumber cotyledons were converted into nucleosides by the combined action of snake venom phosphodiesterase and alkaline phosphatase and separately fractionated on Sephadex LH-20 column (46×1 cm) using 35% ethanol for elution. Authentic samples of i^6A and io^6A were separately chromatographed on the same column. Fractions corresponding to those of authentic samples of i^6A and io^6A were pooled, evaporated to dryness, dissolved in TBS and used for estimation by radioimmunoassays using specific anti-i^6A and anti-io^6A antibodies by comparing binding inhibition with appropriate standard curves.

Analysis of spot no. 5

Spot 5, on treatment with spleen phosphodiesterase, produced in equimolar amounts, Up and a second nucleotide migrating just below the origin (figure 4, lane b). It could be either m^1Ap or m^7Gp. The identity of the slow-moving spot was established by its change in electrophoretic mobility upon treatment with alkali which affects both nucleotides. Alkali treatment is known to convert m^7Gp to 4-amino-5-(N-methyl)formamide isocytosine ribotide which migrates just ahead of Gp on electrophoresis at pH 3·5. m^1Ap is converted to m^6Ap, which comigrates with Ap (Cory and Adams, 1975). When the slow-moving spot (figure 4, lane b) was treated with alkali, it migrated with Ap (lane d). In addition, the slower moving phosphodiesterase product (figure 4, lane b) migrated towards the cathode during
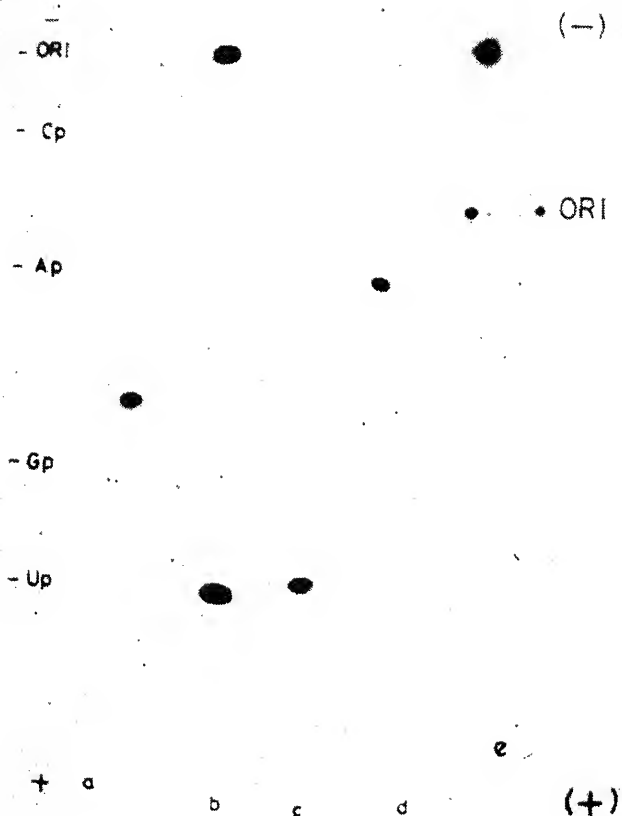
Figure 4. Analysis of the dinucleotide from spot 5 (figure 2). The dinucleotide was eluted and (a) reincubated with RNase T_2; (b) digested with spleen phosphodiesterase; or (c) digested with nuclease P1. In each case the products were analysed by electrophoresis on Whatman No. 3 paper. The slow-moving spot from (b) was eluted and (d) treated with 0·1 N NaOH at 37°C for 16 h and electrophoresed on Whatman No. 3 paper; or (e) electrophoresed on cellulose acetate at pH 3·5.

confirmed by incubation of the eluted dinucleotide with the enzyme followed by electrophoresis (figure 4, lane a).

Determination of the 3′ terminal nucleotide of the dinucleotide spot 5 (figure 2) would indicate which of the two nucleotides (m^1Ap or Up) is 2′-O-methylated. The dinucleotide spot 5 was eluted and digested with nuclease P1. Nuclease P1 has a 3′, 5′-phosphodiesterase activity (Fugimoto *et al.*, 1969). Therefore a structure XmpYp, upon hydrolysis by nuclease P1, would yield the nucleotide at the 3′ end as its 5′ phosphate (pY). The dinucleotide spot no. 5 gave pU as the product of nuclease P1

Discussion

Two-dimensional TLC of $[^{32}P]$tRNA digest shows that the spot corresponding to m^2Gp is absent in the light-grown tissue (figure 1b). When the tissue is grown in continuous darkness its tRNA contains m^2G, as revealed by the chromatogram (figure 1a). m^2G has been shown to be present in tRNAs of several plant tissues but is not found in chloroplast with the exception of *Spinacia oleracea* chloroplast tRNA[trp] (Sprinzl *et al.*, 1986). It is interesting that methylation of guanosine to 2-methylguanosine occurs in the dark-grown tissue and that m^2G is absent in the tRNA of light-grown tissue. This indicates that this methylation product may not be of chloroplast origin. However, it is not clear how light suppresses the formation of this nucleotide in the cytoplasm. The chloroplast tRNAs and the synthetases have been shown to be absent in dark-grown cells where chloroplast development is suppressed, but are induced by light (Barnett *et al.*, 1969; Parthier *et al.*, 1972).

In the present studies transfer RNA from dark-grown tissue has been found to contain a high proportion of Tp whereas in the light-grown tissue Tp content of tRNA decreased to 50% (figure 1 and table 1). Similar growth-dependent modifications of Tp in tRNA are known in a few systems. The Tp content of the tRNA of *Dictyostelium discoideum* decreases about 2-fold during development and differentiation (Dingermann *et al.*, 1977). On the contrary, in *Acetabularia mediterrancea* the proportion of Tp increases rapidly by almost 4-fold during development (Schmidt *et al.*, 1977). Light-induced change in the amount of Tp as shown by the present studies is the first report of its kind.

Among modified nucleosides the cytokinins are of unusual interest as they are plant hormones, stimulating cell division and cell differentiation in plant systems (Hall, 1973), and also because they are components of tRNAs of numerous organisms. The result reported in this paper (table 2) established that tRNA of both light- and dark-grown tissues contains both i^6A and io^6A, of which io^6A is the major cytokinin in both the cases. However, tRNA from dark-grown tissue had slightly higher levels of both i^6A and io^6A compared to light-grown tissue.

The relative proportion of ribose methylation is not significantly different in the tRNAs of light- and dark-grown tissues in the present studies although light-grown tissue appears to contain slightly more 2'-O-methylnucleotides (table 1). No significant amount of CmpAp/AmpCp was detected in the dark-grown tissue tRNA, while a substantial amount of these dinucleotides was observed in the light-grown tissue. The significance of the presence of CmpAp/AmpCp in light-exposed tissue is not clear.

Evidence obtained in the present studies shows that 2'-O-methyl-1-methyl-adenosine, a nucleotide modified both in the base and the ribose, is present in the tRNA of cucumber cotyledons. So far, m^1Am has been reported only in the tRNA of ragi (Raviprakash and Cherayil, 1984). However, the effect of light on the amount of this modified nucleotide has not been investigated in these studies.

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# of nuclear proteins from silk glands of *Bombyx mori*

PUSHPA AGRAWAL and K. P. GOPINATHAN*

Centre for Genetic Engineering and Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore 560 012, India

Abstract. A gentle method for the isolation of nuclei from developing silk glands of *Bombyx mori* has been standardized. The nuclei, whether isolated or directly visualized *in situ* within the silk glands, exhibit complex morphology. The nuclei occupy almost the entire volume of the gigantic silk gland cells. Although the isolated nuclei still retain their ramified morphology, being polyploid they are fragile and often become fragmented. The histone and low-salt-extractable proteins from nuclei isolated from the middle and posterior silk glands on different days of the fourth and fifth instars of larval development have been analysed. The histones did not show any stage- or tissue-specific variations whereas the low-salt-extractable proteins showed some developmental stage specific variation. Using the antibody raised against one such protein, its absence in the early stage of development has been confirmed by Western blotting techniques. This developmental stage specific protein may be functionally linked to some activities responsible for boosting up the production of silk or silk-related proteins during the fifth instar of larval development.

Keywords. Nuclei isolation; ramified nuclei; developmental stage specific proteins; histones; silk gland nuclei; silk gland proteins.

n

rm *Bombyx mori* possesses a pair of long, tubular organs called the silk ich are divided into anatomically and functionally distinct regions 077). The silk glands produce the major classes of silk proteins. Fibroin, re protein is synthesized in the posterior silk gland (PSG) (Couble *et al.*, ura *et al.*, 1985), and sericins, a group of adhesive proteins that coat the produced in the middle silk gland (MSG) (Ishikawa and Suzuki, 1985). that encode these proteins are actively expressed in a developmental fic manner mainly during the fifth instar of larval development (Suzuki, lhomme and Couble, 1979).

glands of *B. mori* are fully formed at the end of embryonic development and Kafatos, 1984) and no further cell divisions take place afterwards. the cells grow much larger in size as development progresses. The nuclei d cells undergo dramatic changes in morphology in the course of larval . During larval development, DNA synthesis in the middle and the silk glands continues without cell division. The DNA content of these nuclei increases by about 2×10^5 times over that of the diploid nuclei '4; Tashiro *et al.*, 1968). Many rounds of endomitotic DNA replication

the last 3 instars: an average of 18–19 doublings in the posterior, 19–20 in the middle, and 13 in the anterior silk gland. Due to polyploidization the nuclei of silk gland cells become progressively ramified. In fact, at the middle of the fifth instar an extremely ramified nucleus spreads all over within the cell (Akai, 1983). Fragility, the highly lobate nature of the ramified nuclei and the presence within the cells of a large amount of silk proteins, which are either easily transformed into insoluble masses or coprecipitate with nuclei, pose major difficulties in preparing pure nuclei (Suzuki and Giza, 1976). It requires special care to isolate nuclei of such unusual morphology. We have standardized a simple procedure to isolate the nuclei in sufficient purity. The pure preparations of nuclei were used to analyse the histone- and low-salt-extractable proteins. Electrophoresis of the extracted proteins from middle and posterior silk gland nuclei on different days of the fourth and fifth instars was carried out to examine any tissue and developmental stage specific variations.

Materials and methods

The silk worm *Bombyx mori* NB_4D_2 strain was used for all the experiments. The biochemicals and reagents were from the Sigma Chemical Company, St Louis, Missouri, USA.

The PSG and MSG from larvae in the late third instar and on all days of the fourth and fifth instars were examined. The excised glands were briefly washed in ice-cold KCl (100 mM), rapidly frozen in liquid nitrogen and stored at $-90°C$.

Staining of nuclei located within the silk glands

The frozen glands of the late third or fourth instar were thawed in glycerine–Hanks solution (1:1, v/v) (Ichimura *et al.*, 1985) and of the fifth instar in Hanks solution (0·14 M NaCl, 5·4 mM KCl, 0·8 mM $MgSO_4$, 0·9 mM $CaCl_2$, 3 mM KH_2PO_4, 3 mM Na_2HPO_4 and 0·1% glucose, pH 6·1). The thawed glands were incubated with a few drops of collagenase (Worthington Biochemicals, 0·5 units/ml) in Hanks solution, pH 7·2, for 5 h at 37°C. The glands were then stained with orcein (0·4% in 90% ethanol), rinsed once with 45% acetic acid, mounted on a glass slide under a cover slip in 50% glycerol, and observed under bright field. The photographs were taken using a Zeiss transmitted light photomicroscope. A similar set of glands treated with collagenase was stained with acridine orange (10 μg/ml in PBS) for 15 min at room temperature, washed 4–5 times with PBS and mounted on a glass slide in 50% glycerol in PBS. Fluorescence micrographs were taken (Adams and Kamentsky, 1971) using a Zeiss epifluorescence condensor III RS D 7082 fluorescence microscope.

Isolation of nuclei

The frozen PSG and MSG of all days of the fourth and fifth instars were thawed in

eae) remain on the nylon mesh. In the case of PSG the samples were kept
for about 4 h to denature and coagulate the fibroin prior to filtration.
lasm present in the filtrate was removed by repeated suspension and
n in Hanks solution until the crude nuclei were left as a sediment in clear
ution. Nuclei were treated with 0·5% Nonidet P-40 (NP-40) and centri-
00 *g* for 2 min in a swinging bucket rotor. The pellet was suspended in
ffer (10 mM Tris-HCl, pH 7·8, 3 mM MgCl$_2$, 150 mM KCl, 0·5 mM
itol) containing NP-40, stirred for 30 min and filtered through a very fine
h (pore size 0·1 mm). This crude preparation was applied on the top of a
ent comprising of 40% (10 ml) and 80% (3 ml) Percoll in TMK buffer
al., 1987) and centrifuged at 600 *g* for 20 min. The nuclei located at the
were again applied on a second gradient consisting of 50% and 80%
he nuclei from the interface were checked for purity and the presence of
lasmic material by fluorescence microscopy after staining with 0·01%
range in PBS.

NA and RNA contents of the preparation were quantitated by the
mine reaction (Burton, 1956) and the orcinol reaction (Ceriotti, 1955)
ly. Protein was estimated according to Lowry *et al.* (1951).

f the nuclear proteins

ed nuclei from MSG and PSG of different days of the fourth and fifth
re treated twice with low concentration of salt (0·25 M NaCl) and 0·1 mM
thylsulphonyl fluoride (PMSF) to extract the non-histone proteins. This
ved by extraction of histone proteins using dilute acid (0·25 N HCl). The
nd low-salt-extractable proteins were analysed by electrophoresis on
amide gels containing sodium dodecyl sulphate (SDS). The histone
extracted with 0·25 N HCl) were separated on 12·5% SDS–polyacrylamide
stained with 0·25% Coomassie brilliant blue-G (CBB) in 40% methanol
acetic acid. The 0·25 M NaCl extracted proteins were separated on 9%
acrylamide gels. These gels were first stained with 0·25% CBB and then
ver staining procedure (Morrissey, 1981).

s to a specific protein

to raise antibodies against a single protein appearing in a developmental
ific manner, preparative SDS–polyacrylamide slab gel electrophoresis was
ut on 9% gels. About 3–4 mg of total low-salt-extractable proteins from
r silk gland nuclei were loaded onto the gel. Electrophoresis of nuclear
was carried out at constant voltage (80–100 V). The gel was fixed in 5%

administered at weekly intervals. A fifth injection was given subcutaneously
finally a booster injection using the solubilized protein eluted from acrylamide
slice. Blood (10–15 ml) was collected through the marginal ear vein 5–6 days
the final injection and allowed to clot overnight in a refrigerator. The serum
clarified by centrifugation (3250 g, 10 min) and was tested for the presence
antibody by the Ouchterlony double diffusion method as well as the more sens
Western blotting technique.

(A)

(B)

(D)
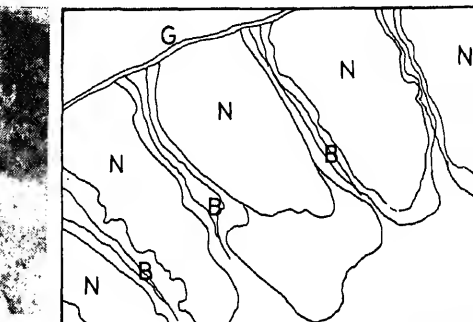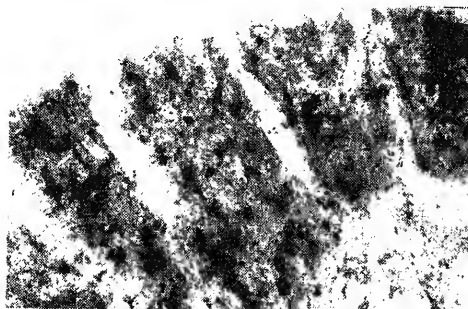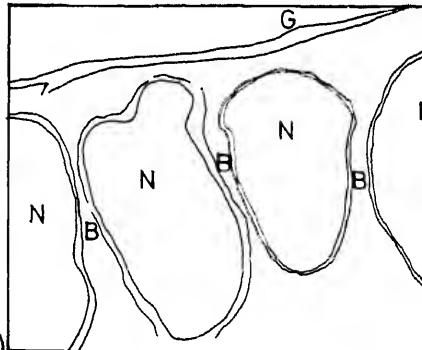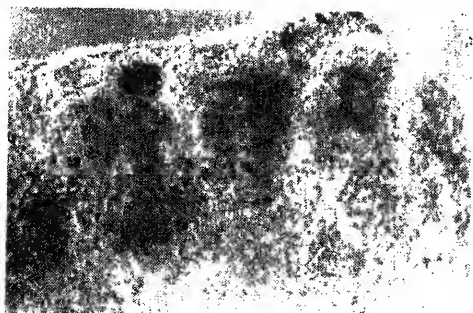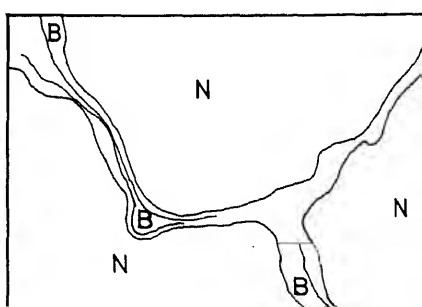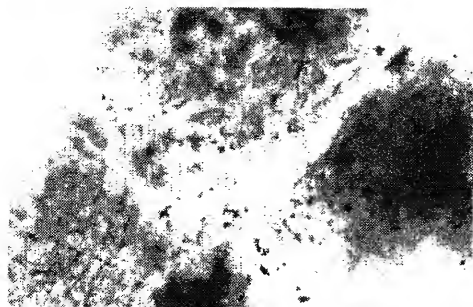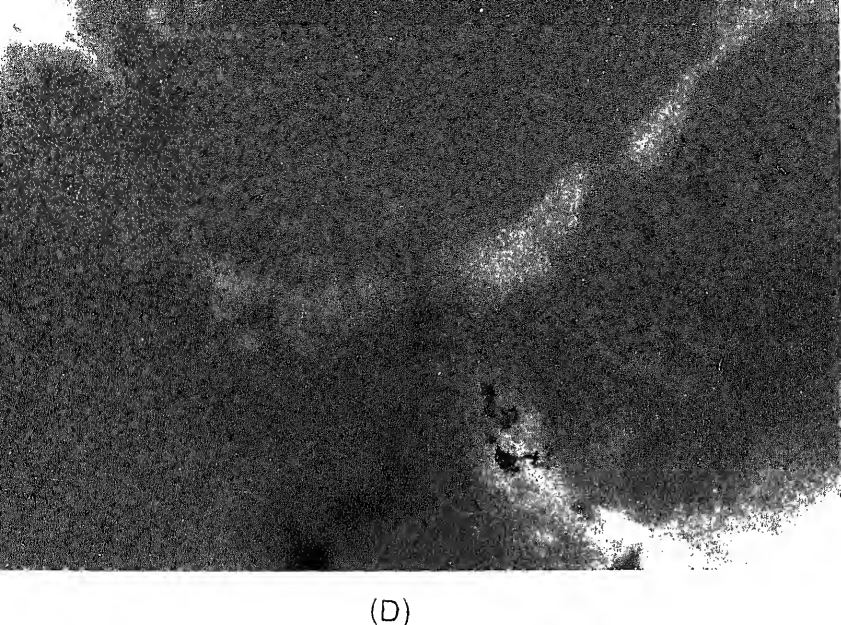
Figure 1. Visualization of nuclei within the silk glands. Bright field micrographs of orcein-stained PSG cell nuclei of (A) fourth day of third instar, (B) third day of fourth instar, and (C) fourth day of fifth instar. (D) Same as C, shown in colour. Line drawings are also provided for clarity. Magnification is the same in all the photographs. N, nucleus; B, cell boundary; G, silk gland boundary.

blotting

hod described by Towbin *et al.* (1979) was used with some modifications. salt (0·25 M NaCl) extracted proteins from nuclei isolated from fourth and ars silk glands were separated on a 9% polyacrylamide gel under de-conditions. The samples were included in duplicate in two separate and after the run the gel was cut longitudinally into halves. One half was vith CBB and the other was electrophoretically blotted for 12–16 h onto ulose filter. The transferred proteins on the nitrocellulose filter were probed iserum raised against the 50 kDa protein seen in extracts from nuclei of ar glands. The filter was first soaked in PBS containing 2–3% BSA and veen 20, incubated for 2–3 h at room temperature and washed 3 times each) with PBS containing 0·05% Tween 20, on a shaker. The filter was ibated for 2 h at 37°C with the antiserum (diluted 1:10 in PBS, containing ween 20). Subsequently the filter was washed with PBS–Tween for 1 h with s of buffer and incubated for 1 h with goat antirabbit IgG–horseradish se (HRPO) conjugate. The filter was washed thrice in PBS–Tween and PBS and then incubated for 10 min in 10 ml of citrate buffer containing oenzidine (10 mg), CoCl₂ (0·1 ml of 1% solution) and 7·5 µl of 30% H₂O₂
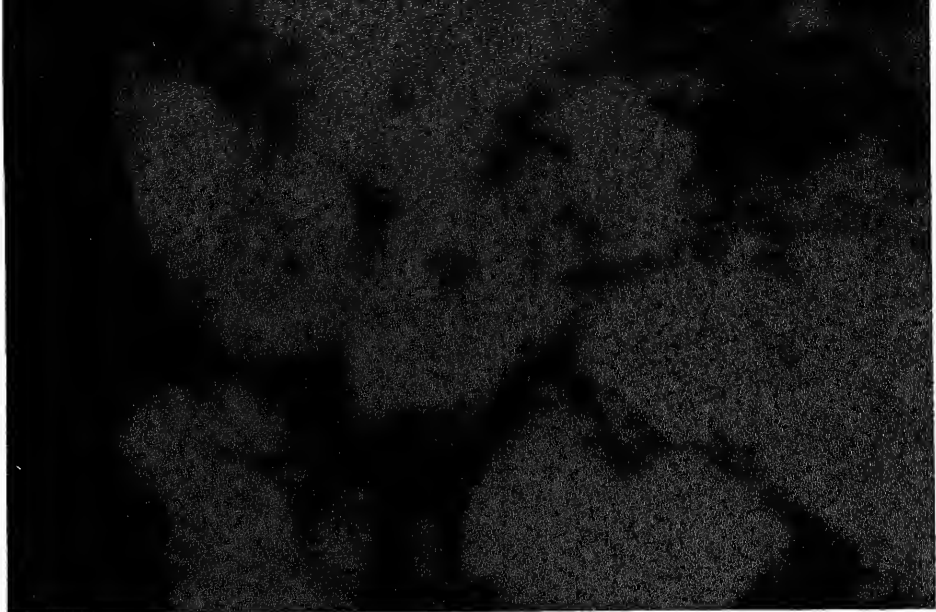
Figure 3. Isolated nuclei. The nuclei isolated from fifth instar PSG were stained with acridine orange and examined under fluorescence microscope. (A) The yellow to green fluorescence represents the nuclear material. Absence of red fluorescence confirms that there is no cytoplasmic contamination. N, Nucleus.

to develop the colour reaction (Hsu and Soban, 1982). Appropriate controls with nonimmune serum were always included.

Results

Staining of nuclei within the silk glands

The middle and posterior silk glands of late third and all days of fourth and fifth instars were treated with collagenase to remove connective tissue material and directly stained with orcein or acridine orange. Figure 1 shows the bright field micrographs of orcein-stained nuclei within the silk gland at late third, fourth and fifth instars. The boundaries of the gigantic cells making up the silk glands and the nuclei nearly filling the entire volume of the cells though diffused, can be made out. For clarity line reproductions of the photographs are also provided. The nuclei are elongated in the direction of the long axis of the cells in third instar. Ramification of the nucleus starts in the last days of the third instar (figure 1A) and in the fourth instar many lobes stretch to form a long backbone (figure 1B).

The fluorescence micrographs of acridine orange stained nuclei within the silk gland at fourth and fifth instars are shown in figure 2. For comparison phase
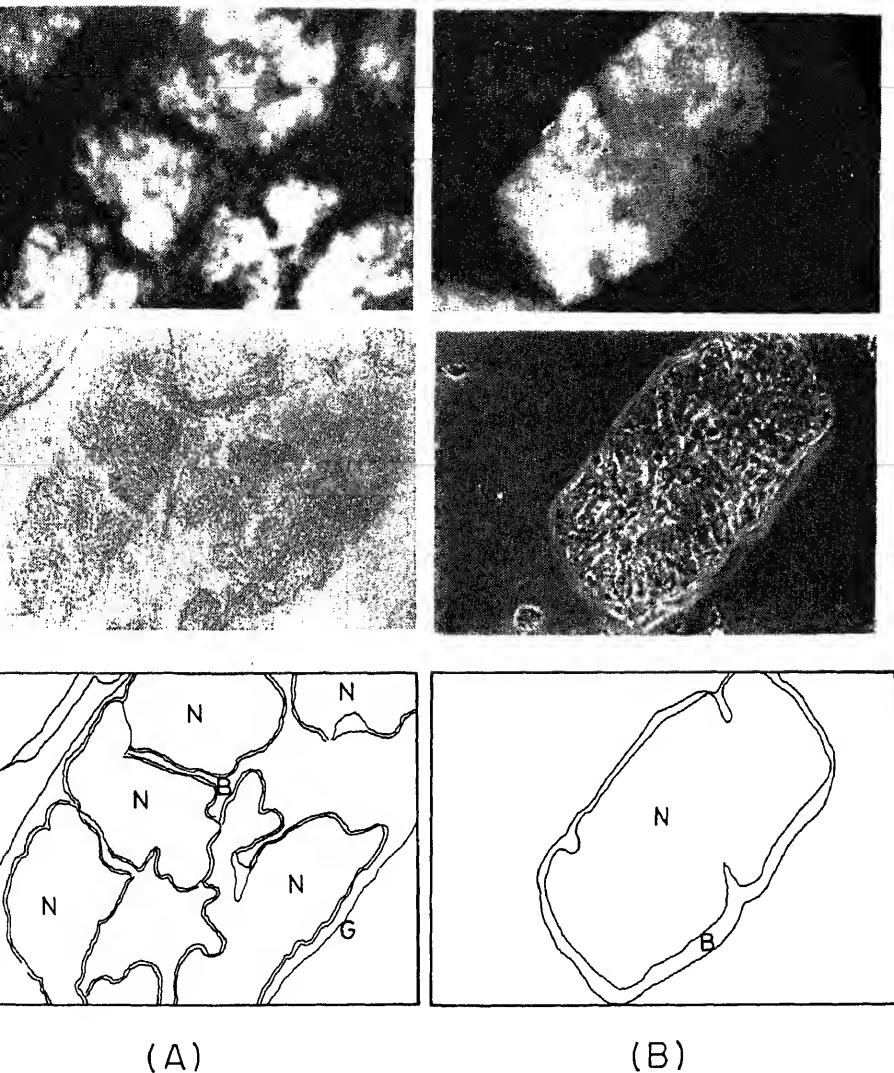
Figure 2. Fluorescence staining of nuclei within the silk gland. Fluorescence micrographs of the acridine orange-stained nuclei within the PSG: (A) fourth day of fourth instar and (B) fifth day of fifth instar. The corresponding phase contrast micrographs and line drawings are shown in lower rows of each column. N, Nucleus; B, cell boundary; G, silk gland boundary.

ei occupy almost the entire cell volume (compare fluorescence with corresponding phase contrast micrograph in figure 2B). The major structures visible in the ds are due to nuclei while the cell boundaries are not very distinct. The ment of the glands with collagenase also makes the cell boundaries diffuse. ever, the gigantic size and the increasing dimensions of the cells in

Isolation of nuclei

The middle and posterior silk glands of different days of the fourth and fifth instars were excised for isolation of nuclei. During isolation, the nuclei get fragmented to some extent even under extremely mild conditions. Although fragments of varying sizes were observed, they retained the ramified morphology. The fluorescence micrographs of the isolated nuclei after acridine orange staining show negligible cytoplasmic contamination, as evidenced by the absence of red fluorescence (figure 3). The nuclei isolated by Kondo *et al.* (1987) had substantial cytoplasmic contamination. Although the basic techniques were similar (see discussion) the method we have utilized yielded better preparations of nuclei. The weight ratio of DNA/RNA/histone proteins/low-salt-extractable proteins was found to be approximately $1:1:1:2.5$, substantiating the purity of the nuclear preparation.

Analysis of the nuclear proteins

The acid-soluble proteins extracted by 0·25 N HCl from nuclei of MSG and PSG of different days of fourth and fifth instar larvae were analysed on 12·5% SDS–polyacrylamide gels. The electrophoresis pattern in figure 4 demonstrates the existence of the 4 core histones and histone H1. There were additional protein bands, presumably other species of histones or modified histones as well as nonhistone proteins present in the acid-extracted samples from both the tissues and
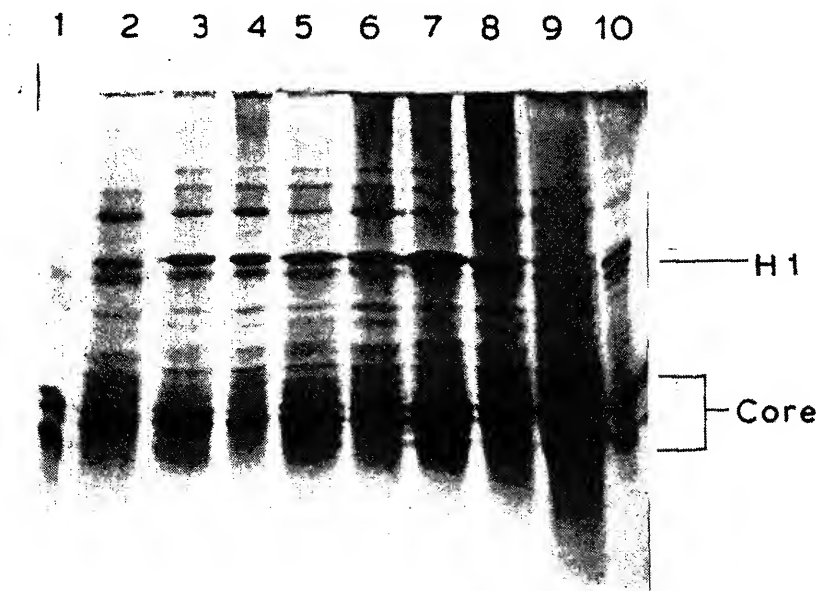
Figure 4. Histones from silk gland nuclei. The histone proteins extracted with 0·25 N HCl from silk gland nuclei of different stages were subjected to electrophoresis on 12·5% SDS–

he stages. By and large the histone composition was similar in MSG and
uclei at all the stages. The additional bands of proteins seen in the histone
re present irrespective of whether the previous extraction was carried out
25 M or 0·35 M NaCl. On the other hand, prior treatment with 0·35 M NaCl
d the histone Hl. Therefore we resorted to using 0·25 M NaCl in all the
uent experiments.

overall pattern of distribution of acid extractable proteins in PSG and MSG
was similar.

electrophoresis pattern of the 0·25 M NaCl extracted proteins on SDS–
rylamide gel is presented in figure 5. A large number of proteins were
ced and some of them were negatively stained with silver when present in
ve amounts. Nevertheless some development stage specific variations are
s in the protein banding pattern. For instance, a protein of about 50 kDa
esent in samples of all days of the fifth instar in both MSG and PSG (arrow
e 5). This band was not traceable in either of the tissues in any fourth instar
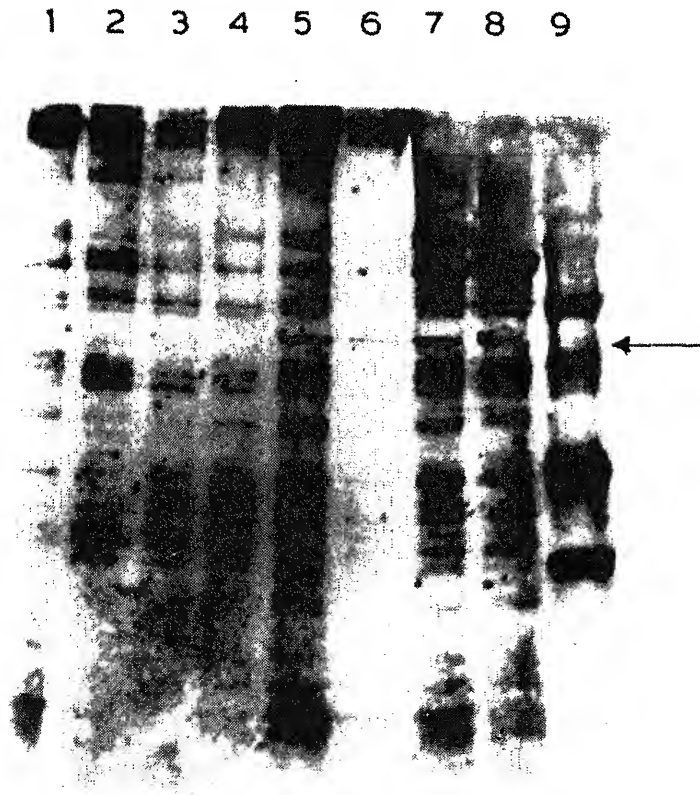
Figure 5. Low-salt-extractable proteins of silk gland nuclei. Proteins of silk gland nuclei
extracted with 0·25 M NaCl were subjected to electrophoresis on SDS–polyacrylamide gels
(9%). The gel was stained with CBB and then by silver staining. Lanes 1–4, fourth instar

sample. Similar variations were seen for some other proteins. The absence of the 50 kDa protein in the fourth instar samples was consistently observed in all the preparations.

Antibodies to the developmental stage specific protein

The 50 kDa protein which was detected in fifth instar silk gland cell nuclei but was conspicuously absent in the fourth instar silk gland nuclei was used for immunizing a rabbit for the production of antibodies. The rabbit serum, collected after the administration of even the booster dose of the specific protein, did not give the precipitin band in the Ouchterlony immunodiffusion test. The failure to demonstrate the presence of specific antibody in the serum by this test led us to the more sensitive Western blotting method. The results of the immunoblotting are presented in figure 6, which shows the specific protein revealed by antibody. This result confirms the presence of specific antibody in the serum. No bands were seen in control blots probed with nonimmune serum. The blot probed with immune serum shows two adjoining bands (figure 6), migrating close to each other. Although one single band of protein was cut out and injected to elicit antibodies, the heterogeneity could have arisen owing to contamination from a neighbouring protein band in the gel. The contaminating band can be visualized as a weak signal in the fourth instar lane also. Most importantly, however, the lane containing the fourth instar sample of nuclear proteins did not show the immunoreactive band corresponding to the 50 kDa protein, confirming its absence. The 50 kDa band could be seen in both MSG and PSG of the fifth instar. Thus, this protein appears to be a developmental stage specific protein present in both the tissue only during the fifth instar.

Discussion

The silk gland of *Bombyx mori* has served as a convenient model system for the study of tissue specific and developmental stage specific gene expression (Suzuki, 1977; Prudhomme and Couble, 1979). The MSG and PSG are made up of approximately 255 and 520 cells respectively (Goldsmith and Kafatos, 1984). During larval development, the cells continue to grow larger in size without division. The nuclei also do not divide; however, the DNA replication continues. As a result, the nuclei grow enormously large in size and become ramified. It has proved to be a formidable task to isolate intact nuclei from the silk gland. Isolation of nearly satisfactory preparations of nuclei from the silk glands have been recently reported (Ichimura *et al.*, 1985; Kondo *et al.*, 1987). Although the method we have developed here for the isolation of nuclei is similar to that of Kondo *et al.* (1987), there are some differences: (i) We have used Hanks solution as starting working medium against TMK buffer used by Kondo *et al.* (1987). (ii) We have repeatedly decanted the nuclear preparation with Hanks solution until the crude nuclei were
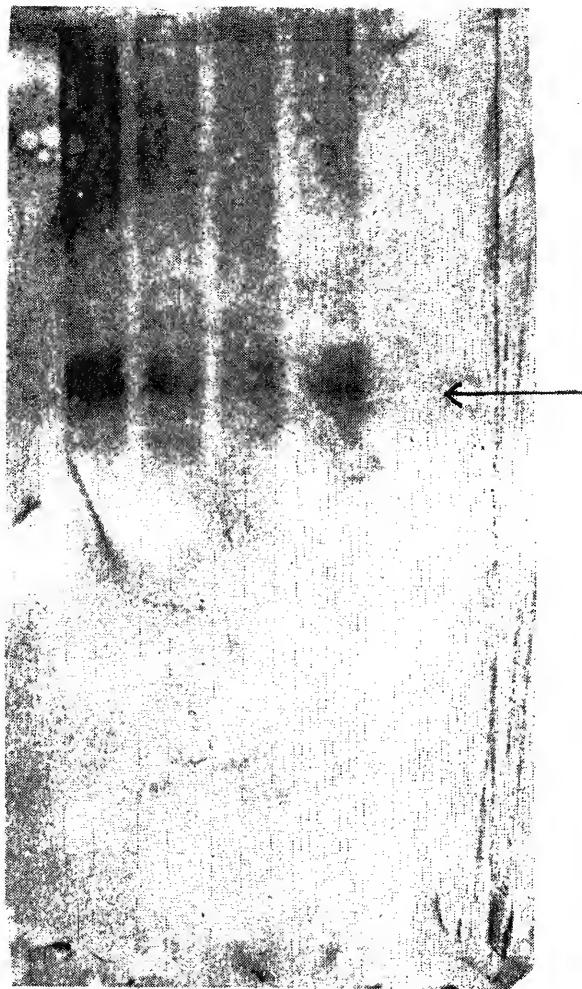
Figure 6. Western blot analysis. Antibodies raised against a single protein band (shown in figure 5) was used to probe the low-salt-extracted proteins from silk gland nuclei. The detection system used was the colour reaction catalysed by HRPO coupled to goat antirabbit IgG. Lane 1, fourth instar fourth day sample; lanes 2–5, fifth instar samples in the order MSG and PSG on third day and MSG and PSG on fifth day respectively; lane 6, standard marker protein. Arrow indicates the position of the 50 kDa protein band.

rations were clean and were devoid of cytoplasmic contamination. However, the fragility of the ramified nuclei was evident even in our preparations.

It was of interest to investigate the origin and the molecular mechanism of the formation of the higher order structure of the giant ramified nuclei and the

due to progressive ramification the nucleus occupies almost the entire cell vol[u...]
by the middle of the fifth instar.

The silk glands of *B. mori* are highly differentiated to produce the silk prote[...]
The production of silk occurs in a tissue-specific and development stage spe[...]
manner. From the reported literature on the control of gene expression in analog[...]
systems, it is evident that the formation of appropriate chromatin structure[...]
necessary for the control of *in vivo* transcription. The association and distributio[...]
proteins on the chromatin are expected to show differences depending on [...]
state—expressing or non-expressing—of the genome. The histone protein patter[...]
MSG or PSG did not show any differences at any stage. The presence of a 50 k[...]
nonhistone protein, however, in both MSG and PSG nuclei only during the f[...]
instar was conspicuous; this protein was absent in the fourth instar. Since i[...]
known that massive silk production starts only towards the end of the fifth ins[...]
the appearance of this protein during the fifth instar may be of significance.
synthesis of silk involves the production of fibroin and sericins, and other access[...]
proteins, as well as the necessary gearing-up of the system. Since the 50 kDa pro[...]
was found in both MSG and PSG, it may not be directly related to fibr[...]
synthesis but rather may be involved in the regulation of expression of any or al[...]
fifth instar protein(s) including the silk-related proteins.

In order to assign a specific function to this 50 kDa protein in silk glands,
have begun by taking an immunological approach to detect it in nuclear extra[...]
Although this protein was not highly antigenic, we could demonstrate the prese[...]
of specific antibodies in serum of immunized rabbit by the sensitive West[...]
blotting method. We propose to use this antibody to isolate and purify the spe[...]
protein(s) from the fifth instar glands by the immunoaffinity procedure. [...]
relation, if any, between the appearance of the 50 kDa protein in a developme[...]
stage specific manner in both MSG and PSG and the synthesis of silk protein[...]
not evident at this point of time.

Acknowledgements

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ANNIE ABRAHAM and P. A. KURUP*

Department of Biochemistry, University of Kerala, Kariavattom, Trivandrum 695 581, India

Abstract. The effect of biotin deficiency on the metabolism of cholesterol was studied in rats fed cholesterol-free and cholesterol-containing diet. Biotin deficiency induced by feeding raw egg-white resulted in higher cholesterol in the serum and aorta, and higher high density lipoprotein cholesterol and low density lipoprotein+very low density lipoprotein cholesterol. In the liver, cholesterol increased only in the cholesterol diet group but not in the cholesterol-free diet group. Levels of triglycerides were lower in the biotin-deficient, cholesterol-free diet group, but triglycerides were elevated in the cholesterol diet group. Concentration of bile acids in the liver and activity of lipoprotein lipase in the heart and adipose tissue were significantly decreased in the biotin-deficient rats. Release of lipoproteins into the circulation, incorporation of $[1,2-^{14}C]$ acetate into cholesterol, and activity of plasma lecithin:cholesterol acyl transferase were higher.

Keywords. Biotin deficiency; lipoprotein lipase; plasma LCAT.

Introduction

During our investigations on the effect of deficiency of vitamins on cholesterol metabolism, it was found that biotin deficiency produced significant hypercholesterolemia in rats fed cholesterol-free and cholesterol-containing diet. Apart from a report by O'Neill and Bannister (1984) on increased cholesterogenesis in hepatocytes isolated from biotin-deficient chicks and an earlier observation of Scott (1958) on the hypercholesterolemia in a boy having biotin deficiency, no other reports seem to be available in this respect. We have studied the mechanism of this hypercholesterolemic action in biotin deficiency and the results are reported in this paper.

Materials and methods

Female albino rats (Sprague-Dawley strain, weight 60–80 g) were grouped as follows with 15 rats in each group, in two separate experiments (A and B).

A. Cholesterol-free diet:
1. Biotin-deficient.
1a. Pairfed control group.

B. Cholesterol-containing diet:
2. Biotin-deficient.
2a. Pairfed control group.

Biotin deficiency was induced in groups 1 and 2 by feeding raw egg-white. The composition of the basal diet (g/100 g) is given below.

Cholesterol-free diet: Corn starch, 67; casein (vitamin- and fat-free), 5; egg-white, 15; groundnut oil, 8; salt mixture, 4; vitamin mixture (without biotin), 1.

Cholesterol-containing diet: Corn starch, 58; casein, 5; egg-white, 15; coconut oil, 15; salt mixture, 4; vitamin mixture (without biotin), 1; cholesterol, 2.

Wesson's salt mixture was used (Oser, 1965). $ZnCl_2$ and $CoCl_2 \cdot 6H_2O$ were also added to the diet at a concentration of 15 and 0·15 mg/kg diet respectively. Vitamin mixture used contained (per 100 g diet): retinyl palmitate, 1000 IU; ergocalciferol, 200 IU; α-tocopherol, 12 mg; menadione, 0·3 mg; thiamine hydrochloride, 1 mg; riboflavin, 1 mg; pyridoxine, 0·6 mg; niacin, 10 mg; calcium pantothenate, 5 mg; inositol, 20 mg; choline, 300 mg; folic acid, 0·2 mg; vitamin B_{12}, 3 μg; *p*-amino-benzoic acid, 5 mg; made up to 1 g with dextrose. No biotin was added.

Rats in groups 1 and 2 received raw egg-white while those of the other groups received the same quantity of boiled egg-white. Since diet consumption in the biotin-deficient groups was lower, pairfed control groups were maintained which received the same amount of biotin-adequate diet. The pairfed control rats received 5 μg of biotin/100 g body weight per day subcutaneously. No biotin was given to the deficient groups. The rats were housed individually in polypropylene cages in rooms maintained at $25 \pm 1°C$. Water was available *ad libitum*. The duration of the experiment was 75 days. At the end of this period, the rats were deprived of food overnight, stunned by a blow at the back of the neck, and killed by decapitation. Blood and tissues were removed to ice-cold containers for various estimations.

Estimation of biotin in the serum and liver was carried out microbiologically using *Lactobacillus plantarum* as described by Wright and Skeggs (1944) and Baker *et al.* (1962). Total cholesterol and triglycerides in the serum and tissues were determined as described earlier (Menon and Kurup, 1976). For assay of plasma lecithin:cholesterol acyl transferase (LCAT, EC 2·3·1·43), plasma from heparinized blood was immediately extracted with acetone:ethanol (1:1). Another aliquot was incubated at 37°C for 3 h, at the end of which it was extracted with acetone:ethanol (1:1). Ester cholesterol and unesterified cholesterol were estimated in the lipid extract by the procedure of Schoenheimer and Sperry (1934) and Sperry and Webb (1950). Activity of lipoprotein lipase (EC 3·1·1·3) of the heart and adipose tissue was determined by the method of Krauss *et al.* (1973).

Release of lipoproteins into the circulation was measured using Triton WR 1339 and the estimation of bile acids in the liver was carried out using 3α-hydroxysteroid dehydrogenase as described before (Jaya and Kurup, 1987). Separation of serum lipoproteins into high density lipoprotein (HDL) and very low density lipoprotein (VLDL)+low density lipoprotein (LDL) was carried out as described by Warnick and Albers (1978). *In vivo* incorporation of [1,2-^{14}C]acetate into cholesterol in the liver was carried out as described before (Thomas *et al.*, 1983). Five microcuries of labelled acetate/100 g body weight were administered to the rats.

l raw egg-white and given no biotin developed biotin deficiency and l characteristic symptoms like dermatitis, scaly pigmentation and loss of ese symptoms were more visible in the cholesterol-free diet group. Avidin n the raw egg-white forms a stable complex with biotin which is then not l. Rats in the other groups received the same amount of boiled egg-white, the avidin is inactivated. This was done to ensure that the protein source same in all the groups.

eight gain was lower in biotin-deficient rats ($68 \pm 1 \cdot 7$ g) when compared to the pairfed controls ($75 \pm 2 \cdot 1$ g) in the cholesterol-free diet group. The nding values for the cholesterol diet group were $80 \pm 2 \cdot 1$ and $90 \pm 2 \cdot 3$ g ely. Rats of the deficient groups showed significantly lower biotin levels in m and liver when compared to the corresponding pairfed controls. The ere $0 \cdot 65 \pm 0 \cdot 016$ and $1 \cdot 10 \pm 0 \cdot 028$ (μg/100 ml) for serum and $30 \cdot 82 \pm 0 \cdot 74$ and $\cdot 55$ (μg/100 g wet tissue) for liver in the biotin-deficient and pairfed rats ely for the cholesterol-free diet group. The corresponding values for the ol diet group were $0 \cdot 83 \pm 0 \cdot 023$ and $1 \cdot 30 \pm 0 \cdot 035$ for serum and $42 \cdot 55 \pm 1 \cdot 10$ $0 \pm 2 \cdot 10$ for liver.

ation of cholesterol and triglycerides

eficient rats of the cholesterol-free diet group showed higher cholesterol in d aorta when compared to the corresponding pairfed controls. But liver ol was not significantly altered. Our results on liver cholesterol are in nt with the report of absence of any significant change by Curran (1950))akshinamurti and Desjardins (1968) in biotin deficiency. In the cholesterol up, the cholesterol in serum, liver and aorta showed significant increase mpared to the levels in the corresponding pairfed controls. Biotin deficiency l both HDL cholesterol and LDL + VLDL cholesterol in both the groups. usion of cholesterol in the diet attenuated the increase in the cholesterol serum in biotin deficiency. Liver cholesterol, which was not significantly n the cholesterol-free diet, was increased in the cholesterol diet in biotin y. Triglyceride levels were lower in serum, liver and aorta in the biotin- rats in the cholesterol-free diet group. In the cholesterol diet group, biotin-deficient rats showed elevated triglycerides in these tissues (tables 1

Table 1. Concentration of cholesterol and triglycerides in serum, liver and aorta of biotin-deficient and normal rats fed cholesterol-free and cholesterol-containing diet.

Group	Serum (mg/100 ml)		Liver (. . . . . . ml/100 g wet tissue . . . . . .)		Aorta	
	Chl	Tgls*	Chl	Tgls*	Chl	Tgls*
A. Cholesterol-free diet						
1. Biotin-deficient	79·50 ± 2·23[a]	5·23 ± 0·14[a]	405·65 ± 11·35	334·68 ± 8·7[a]	249·60 ± 6·98[a]	598·5 ± 14·96[a]
1a. Pairfed control	64·84 ± 1·75	6·75 ± 0·20	385·45 ± 10·75	395·85 ± 11·5	189·50 ± 5·12	708·3 ± 20·54
B. Cholesterol diet						
2. Biotin-deficient	195·30 ± 5·47[a]	14·75 ± 0·43[a]	1620·65 ± 42·14[a]	1215·5 ± 31·6[b]	426·80 ± 11·95[b]	1768·0 ± 49·5[a]
2a. Pairfed control	151·85 ± 3·80	12·80 ± 0·32	1345·75 ± 32·30	998·8 ± 23·9	385·65 ± 9·25	1505·0 ± 40·6

*Triglycerides are expressed as triglyceride glycerol.

Values are mean (n = 6) ± SEM.

Significance of difference: group 1 vs group 1a; group 2 vs group 2a.

[a] $P < 0.01$; [b] $0.01 < P < 0.05$.

Chl, Cholesterol; Tgls, triglycerides.

Table 2. Incorporation of labelled acetate into hepatic cholesterol, concentration of hepatic bile acids, and activity of lipoprotein lipase in biotin-deficient and normal rats fed cholesterol-free and cholesterol-containing diet.

Group	*In vivo* incorporation of [1,2-^{14}C] acetate into hepatic cholesterol (cpm/mg protein)	Hepatic bile acids (mg/100 g wet tissue)	Lipoprotein lipase (μmol glycerol h/g protein) Adipose tissue	Heart
A. Cholesterol-free diet				
1. Biotin-deficient	$9{\cdot}06 \pm 0{\cdot}24^a$	$22{\cdot}35 \pm 0{\cdot}60^a$	$128{\cdot}55 \pm 3{\cdot}21^a$	$25{\cdot}05 \pm 0{\cdot}65^a$
1a. Pairfed control	$6{\cdot}66 \pm 0{\cdot}18$	$30{\cdot}60 \pm 0{\cdot}88$	$146{\cdot}02 \pm 4{\cdot}09$	$31{\cdot}48 \pm 0{\cdot}92$
B. Cholesterol diet				
2. Biotin-deficient	$5{\cdot}25 \pm 0{\cdot}15^a$	$35{\cdot}60 \pm 0{\cdot}89^a$	$105{\cdot}85 \pm 2{\cdot}65^a$	$17{\cdot}95 \pm 0{\cdot}47^a$
2a. Pairfed control	$3{\cdot}79 \pm 0{\cdot}10$	$43{\cdot}25 \pm 1{\cdot}17$	$122{\cdot}50 \pm 3{\cdot}43$	$23{\cdot}84 \pm 0{\cdot}67$

Details as in table 1.

Table 3. Concentration of cholesterol in serum lipoprotein fractions and release of lipoproteins into the circulation in biotin-deficient and normal rats fed cholesterol-free and cholesterol-containing diet.

Group	HDL (mg/100 ml serum)	LDL + VLDL	Release of lipoproteins into the circulation Concentration of cholesterol (.... mg/100 ml serum ......) Saline-injected group	Triton-injected group	Increase in cholesterol (%)
A. Cholesterol-free diet					
1. Biotin-deficient	$59{\cdot}60 \pm 1{\cdot}67^a$	$19{\cdot}75 \pm 0{\cdot}55^a$	$80{\cdot}65 \pm 2{\cdot}09$	$230{\cdot}6 \pm 6{\cdot}45$	$186{\cdot}0 \pm 5{\cdot}0^a$
1a. Pairfed control	$48{\cdot}53 \pm 1{\cdot}16$	$14{\cdot}83 \pm 0{\cdot}38$	$63{\cdot}88 \pm 1{\cdot}47$	$155{\cdot}23 \pm 3{\cdot}73$	$143{\cdot}0 \pm 3{\cdot}4$
B. Cholesterol diet					
2. Biotin-deficient	$55{\cdot}70 \pm 1{\cdot}56^a$	$137{\cdot}30 \pm 3{\cdot}98^a$	$193{\cdot}60 \pm 5{\cdot}03$	$478{\cdot}25 \pm 13{\cdot}39$	$147{\cdot}0 \pm 4{\cdot}3^a$
2a. Pairfed control	$41{\cdot}32 \pm 1{\cdot}07$	$112{\cdot}08 \pm 2{\cdot}91$	$150{\cdot}95 \pm 4{\cdot}07$	$341{\cdot}15 \pm 9{\cdot}21$	$126{\cdot}0 \pm 3{\cdot}3$

Details as in table 1.

Activity of lipoprotein lipase

The activity of lipoprotein lipase in heart and adipose tissue showed significant decrease in the biotin-deficient rats when compared to the corresponding pairfed controls. But the activity of plasma LCAT was more in the biotin-deficient rats fed cholesterol-free diet. Activity expressed as per cent increase in the ratio of ester cholesterol to free cholesterol during incubation was $35{\cdot}20 \pm 1{\cdot}03$ in the biotin-deficient rats compared to $27{\cdot}35 \pm 0{\cdot}74$ in the pairfed controls (table 2).

Release of lipoproteins into the circulation

Discussion

The main role of biotin in lipid metabolism is to act as a cofactor for carboxylases, particularly acetyl-coenzyme A (CoA) carboxylase, which catalyses the rate-limiting step in fatty acid synthesis. The decreased concentration of triglycerides in serum and liver in the biotin-deficient rats of the cholesterol-free diet group may be due to the block in the carboxylation of acetyl-CoA to malonyl-CoA. The increase in the serum and liver triglycerides in the biotin-deficient rats of the cholesterol diet group may be due to the high intake of dietary fat. Increased cholesterogenesis, as evidenced by the increased incorporation of labelled acetate into hepatic cholesterol, in the biotin-deficient rats may be due to the fact that more acetyl-CoA (whose utilization for fatty acid synthesis is now decreased) may be diverted for cholesterol synthesis. The fact that liver cholesterol is not significantly different in the cholesterol-free diet group inspite of the increased synthesis of cholesterol in biotin deficiency may be due to the fact that most of the newly synthesized cholesterol is used for lipoprotein synthesis. This is evident from the increased release of lipoproteins into the circulation. This increased release of lipoproteins may also contribute to the hypercholesterolemia and hypertriglyceridemia in biotin deficiency. The increase in aortic cholesterol in the biotin-deficient rats may be due to the increase in the serum LDL + VLDL cholesterol. The major source of cholesterol for the arterial tissue is circulating lipoproteins, particularly LDL.

Hepatic degradation of cholesterol to bile acids is also decreased in the biotin-deficient rats, as indicated by the decreased concentration of bile acids in the liver. This may be due to decreased activity of propionyl-CoA carboxylase, a biotin-dependent enzyme which catalyses the oxidation of propionic acid formed during the conversion of cholesterol to bile acids. The decreased activity of lipoprotein lipase, which is involved in the uptake of circulating triglyceride-rich lipoproteins (chylomicron + VLDL), may result in decreased uptake of these lipoproteins and this, along with the high intake of fat, may contribute to the hypertriglyceridemia in the biotin-deficient rats of the cholesterol diet group. The increase in the activity of LCAT, which is involved in the esterification of free cholesterol in the serum, in the deficient rats may be due to the fact that the substrate for this enzyme, HDL cholesterol, is increased in the deficient rats.

Thus the hypercholesterolemia observed in biotin deficiency may be due to increased synthesis of cholesterol in the liver, decreased breakdown of hepatic cholesterol to bile acids, increased release of lipoproteins into the circulation and their decreased uptake by the extrahepatic tissues.

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ization of rat vaginal epithelium IV. Modulation of transglu-
se activity by oestradiol

S. VIJAYASARADHI* and P. D. GUPTA†

Centre for Cellular and Molecular Biology, Hyderabad 500 007, India
*Present address: Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York 10021, USA

Abstract. Calcium-dependent transglutaminase activity was found to be present in vaginal homogenates from adult cycling rats. Treatment of immature or adult ovariectomized rats with oestradiol (0·1 μg/g body weight) resulted in 1·5–2-fold enhancement in the enzyme activity. Progesterone treatment (0·1 μg/g body weight) decreased the enzyme activity. Analysis of amino acids produced by proteolytic enzyme digestion of insoluble keratins from rat vaginal tissue indicated the presence of γ-glutamyl-ε-lysine dipeptide (4 μmol/g protein) in this protein. These results suggest that oestradiol acts on vaginal tissue and induces the activity of transglutaminase. This enzyme catalyses the formation of γ-glutamyl-ε-lysine crosslinks between keratin polypeptides and thus leads to keratinization of the tissue.

Keywords. Oestradiol; keratinization; transglutaminase.

tion

regular oestrous cycles the vaginal epithelium of rats responds to the
g levels of circulating oestradiol (Vijayasaradhi and Gupta, 1987, 1988).
great deal of information is available on the mode of action of oestradiol in
proliferation of vaginal cells (Ladinsky and Peckham, 1965; Epifanova,
aland et al., 1967) little is known regarding the mechanism of its action in
cellular differentiation (keratinization). Husbands and Walker (1963)
d that the keratinizing influence of oestradiol is confined to the daughter
duced from the basal layer during the period of oestrogenic stimulation.
et al. (1971), Galand and Vandenhende (1973), and Galand and Rognoni
n the other hand, from their studies on the kinetics of [³H]thymidine
ation into various cell layers after oestrogenic stimulation of ovariectomized
ncluded that oestradiol exerts its keratinizing effect by acting on the cells
n the basal layer without an obligatory association with new cell divisions.
of these studies was mainly to identify the target cells for the action of
ol-induced cell differentiation. As yet, however, the molecular mechanism of
ol-induced cell differentiation has not been identified. The most significant
ion in this direction was that of Talwar and Segal (1963) who demonstrated
secondary biological effect of oestradiol in causing proliferation and
tion of vaginal epithelium could be blocked by topical application of
ycin D, thus showing that RNA synthesis might be involved.
l conflicting claims have been made regarding the effects of oestradiol on

reported that epithelial outgrowths of rat and mouse vagina failed to keratinize in response to oestrogens added to the medium. Recently, Gupta *et al.* (1986) showed the presence of oestradiol receptors in cultured vaginal epithelial cells. Iguchi *et al.* (1983) discussed several possibilities, such as changes in oestrogen receptor levels under different culture conditions, for the lack of effect of oestrogens on both growth and keratinization of mouse vaginal epithelial cells. However, oestradiol-induced keratinization of mouse vaginal explants (Hardy *et al.*, 1953; Kahn, 1954; Biggers *et al.*, 1956) and of rat vaginal epithelial cells *in vitro* has been demonstrated (Conti and Tasat, 1986; Gupta *et al.*, 1986; Vijayasaradhi *et al.*, 1987).

Calcium-dependent transglutaminase catalyses the covalent cross-linking of the amino group of primary amines to the carboxyl group of glutamine in protein (Peterson *et al.*, 1983). Though the role of the intracellular and/or extracellular transglutaminases in producing insoluble seminal clots in rodents (Williams-Ashman *et al.*, 1980) and clotting during coagulation of blood (Pisano *et al.*, 1969) has been well studied, the physiological functions of these enzymes in the cell are not well understood. Recent studies on keratinization of epidermal cells in culture have indicated a possible role of transglutaminase in cross-linking of cellular proteins during keratinization (Buxman and Weupper, 1976). Therefore we investigated the possibility of a similar mechanism operating in oestradiol-induced keratinization of vaginal epithelium.

In an attempt to understand the biochemical events that are involved in oestradiol-induced keratinization and involvement of transglutaminase in keratinization, we measured calcium-dependent transglutaminase activity in vaginal homogenates of randomly selected adult rats and assessed the effects of oestradiol or progesterone treatment of intact immature (30-day-old rats with no detectable oestradiol in blood serum) and oestrogen-depleted (ovariectomized) adult animals on transglutaminase activity in vaginal tissue. Amino acid analyses were performed in digests of keratin-enriched vaginal proteins.

Materials and methods

Animals

Female Wistar rats, housed at $25° \pm 2°C$ with a 12 h light: 12 h darkness schedule, and with food and water available *ad libitum*, were used. Oestrous cycles of experimental animals were followed by daily vaginal smears and only those animals showing at least two consecutive cycles were used. Regularly cycling animals were ovariectomized bilaterally and used after 2 weeks. Oestradiol-17β and progesterone (Sigma Chemical Co., St Louis, Missouri, USA), $0.1 \, \mu g/g$ body wt. in 1,2-propanediol, were administered intraperitoneally to 30-day-old intact and adult ovariectomized animals. The animals were killed 12 h after injection. Control

(1 mM). Vaginal tissue was freed from surrounding connective tissues and [homo]genized in a ground-glass homogenizer. The homogenate was centrifuged at [...] g for 30 min at 4°C. The clear supernatant was used either directly or after [...] dialysis against the Tris-HCl EDTA buffer. Enzyme assay was carried out [essenti]ally as described by Leu *et al.* (1982). Briefly, transglutaminase activity was [determ]ined by measuring the amount of [³H]putrescine (19 Ci/mmol) or [...] permidine hydrochloride (100 mCi/mmol) incorporated into crude homogenate [of vag]inal tissue during incubation for various time periods at 37°C. The incuba[tion m]ixture contained 0·1 M Tris-HCl buffer, 2 mM dithiothreitol, 1 mM EDTA, [...]/ml N, N′-dimethylcasein and 10 mM CaCl₂.

[At t]he end of the incubation the reaction was terminated by adding 500 μl of [...] ice-cold TCA and the tubes were left in ice for a further 30 min. The TCA-[insolu]ble precipitates were collected on Whatman GF/C glass fibre filters and [washe]d extensively with ice-cold TCA. Finally each filter was dried with ice-cold [...]thanol and counted for radioactivity in toluene-based scintillation mixture in [Pac]kard liquid scintillation counter.

[Estimat]ion of γ-glutamyl-ε-lysine

[Kerat]in-enriched pellet of vaginal tissue obtained as described in the flow sheet [(figure] 1) was resuspended in Tris-HCl buffer (50 mM, pH 9), containing [mercapt]oethanol (25 mM) and urea (8 M). After incubating overnight at room [tempe]rature, urea-insoluble material was collected by centrifugation (13000 g) in [Eppen]dorf tubes and dried under vacuum. The dried material was digested, [seque]ntially, with pronase (2%), carboxypeptidase A (2%) and carboxypeptidase B

[...]. Flow sheet for preparation of keratin-enriched fraction.

Tissue homogenate in buffer 1
Centrifuged at 10000 g, 15 min at 4°C (Step I)

[Supernat]atant 1	Pellet
	Repeated Step I
[Supernat]atant 1a (pooled 1 and 1a)	Pellet
[Sa]lt-soluble	Suspended and stirred in buffer 2 for 30 min.
	Centrifuged at 10000 g for 30 min (Step II)
[Supernat]atant 2	Pellet
	Repeated Step II
[Supernat]atant 2a (pooled 2 and 2a)	Pellet
[Deterg]ent-soluble	Suspended and homogenized in buffer 3. Centrifuged
	at 10000 g for 30 min (Step III)
[Supernat]atant 3	Pellet
	Repeated Step III
[Supernat]atant 3a (pooled 3 and 3a)	Pellet
[Sa]lt-soluble	Washed several times with distilled water. Centrifuged

(0·5%), leucine amino peptidase (2%) and finally with pronase as described by Murayama *et al.* (1983). These enzymes were purchased from Sigma Chemical Co, St Louis, Missouri, USA. Amino acid analysis of the digest was carried out on a Beckman 119 CL amino acid analyser using a Beckman W3P resin column (23 × 6 mm). Deproteination of the digest was carried out as described by Mondino *et al.* (1972). γ-Glutamyl-ε-lysine (BA Chem. Frienkemiklen, FRG) was used as standard dipeptide for amino acid analysis.

Results

The presence of transglutaminase activity in the homogenates of total vaginal tissue obtained from a randomly selected population of rats is shown in figure 1. The activity was measured as the amount of labelled putrescine or spermidine cross-linked to TCA-precipitable casein in 30 min at 37°C. This represents a true tissue transglutaminase and this was further confirmed by its dependence on calcium and its almost complete inhibition by cadaverine, a specific inhibitor of transglutaminase (table 2).
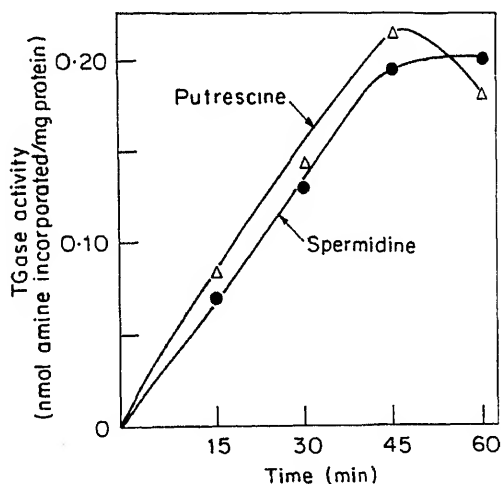
Figure 1. Transglutaminase activity in vaginal protein of randomly selected normally cycling rats. Incorporation of [³H]putrescine (△) and [¹⁴C]spermidine (●) into N, N′-dimethylcasein was plotted as nmol/mg protein as a function of incubation time. Each point was obtained from a pooled (5 animals) sample.

In order to establish whether the activity of transglutaminase in vaginal tissue is hormone-induced, we studied the effect of hormonal treatment on the activity of the enzyme in the homogenate of vaginal tissue from 30-day-old intact and adult chronically ovariectomized rats injected with oestradiol, progesterone or vehicle alone (propanediol). The mean enzyme activity in vehicle-injected control (30-day-

Effect of cadaverine, ionic strength and divalent cations other than Ca^{2+} on transglutaminase n rat vaginal tissue.

t	Transglutaminase activity	
	pmol/mg/h	Control (%)
medium* + tissue protein	250	100
medium + tissue protein + cadaverine (1 mM)	37	15
medium + tissue protein + EGTA (1 mM)	50	20
medium + tissue protein + NaCl (0·15 M)	150	60
without $CaCl_2$ + tissue protein + $MgCl_2$ (1 mM)	67	27
without $CaCl_2$ + tissue protein + $MgCl_2$ (5 mM)	74	30
without $CaCl_2$ + tissue protein + $MnCl_2$ (1 mM)	148	60
without $CaCl_2$ + tissue protein + $MnCl_2$ (5 mM)	125	50

issue protein was incubated in 500 μl of complete assay mixture consisting of 0·1 M Tris-HCl mM dithiothreitol, 1 mM EDTA, 0·2 mg/ml N,N'-dimethylcasein, 1 mM $CaCl_2$, and labelled e (1 μCi/assay) for 45 min at 37°C.
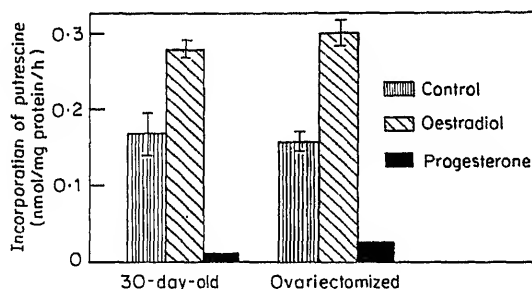
Figure 2. Effect of administration of oestradiol and progesterone on vaginal transglutaminase activity. Enzyme activity was measured 12 h after an intraperitoneal injection (0·1 μg/g body weight) of hormones in propanediol to 30-day-old intact or ovariectomized rats. Controls were injected with an equivalent volume of the vehicle. Values are means ± SEM for 6 animals in each group.

red to the controls. However, progesterone treatment for the same time ed the activity (figure 2).

assess the role of transglutaminase in keratinization we investigated the e of γ-glutamyl-ε-lysine cross-link in keratin-rich vaginal homogenates. tive proteolytic digestion of an urea (8 M) insoluble keratin-enriched fraction combination of enzymes followed by amino acid analysis of the digest were out. γ-Glutamyl-ε-lysine isopeptide cross-link is resistant to proteolytic e. The presence of the dipeptide cross-link is therefore suggestive evidence
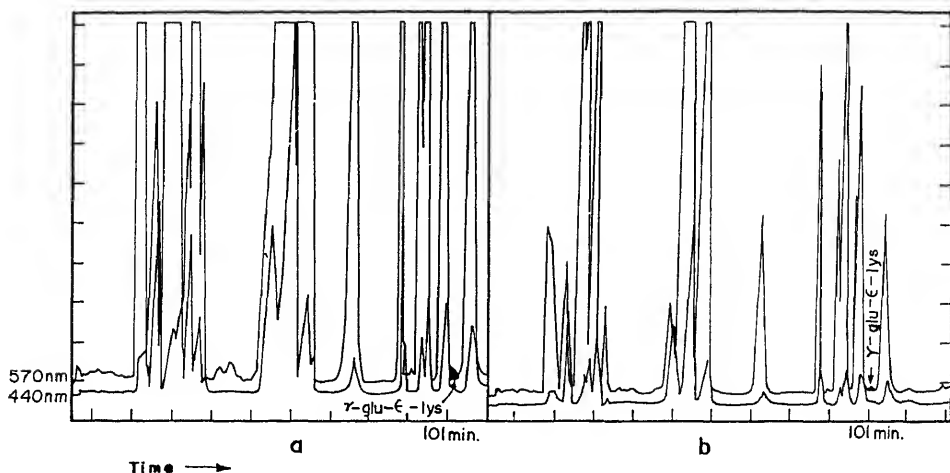
Figure 3. Chromatogram of proteolytic digest of urea (8 M) insoluble vaginal keratins. (a) Separation of standard dipeptide from a standard amino acid mixture containing 50 mmol of each amino acid and of dipeptide. A small peak (arrowed) of dipeptide cross-link of γ-glutamine-ε-lysine was observed at 101 min in 570 nm tracings. (b) Proteolytic digest of 8 M urea insoluble vaginal keratins. Elution from Beckman W3P resin column (23 × 6 mm) with buffer A, 0·2 M lithium citrate (pH 2·83), for 66 min; buffer B, 0·2 M lithium citrate (pH 3·7), for 34 min; and buffer C, 1 M lithium citrate (pH 3·75), for 10 min at a flow rate of 44 ml/h and ninhydrin at 22 ml/h. Constant temperature (40°C) was maintained throughout the run. Here also at 101 min a very small peak (black dot) was seen in the 570 nm tracing.

Discussion

Rice and Green (1977) and Sun and Green (1978) reported that cells in the basal, spinous and granular layers of epidermis synthesize, modify and accumulate the structural proteins required to form the cell envelopes. During keratinization these proteins undergo a process of stabilization by covalent cross-linking that renders them highly insoluble. The absence of a method to separate the epithelium alone from the rest of the underlying connective tissue (as is possible in epidermis) has restricted our biochemical analyses to total tissue free from blood. Our observations are related to oestradiol-induced changes in the total vaginal tissue, which may not however be specific to the epithelial cells alone.

Transglutaminase activity was detected in homogenates of vaginal tissue. This activity appeared similar to that of other intracellular and extracellular transglutaminases reported previously by several authors (Chung and Folk, 1972; Buxman and Weupper, 1976; Leu et al., 1982; Goldsmith, 1983). For example, cadaverine, a specific inhibitor of calcium-dependent transglutaminase activity, inhibited vaginal transglutaminase almost completely. The divalent ions Mg^{2+} and Mn^{2+} were ineffective in maintaining the activity while EGTA, a specific chelator of

transglutaminase of the type present in male coagulating glands and ventral prostate is absent in ovary, uterus and vagina of female rats.

The present investigation implies that transglutaminase activity in vaginal tissue may be regulated by levels of oestradiol. Treatment of 30-day-old intact and ovariectomized animals with oestradiol leads to a significant enhancement in transglutaminase activity. Progesterone treatment is not only ineffective but also results in a decrease in the transglutaminase activity. It may be due to the fact that progesterone acts as an antagonist to oestradiol (Takeda, 1988).

Finally, the presence of small but detectable amounts of the dipeptide γ-glutamyl-ε-lysine in urea (8 M) insoluble proteins of vaginal tissue gives further support for the role of transglutaminase in oestradiol-induced keratinization. Human stratum corneum, which also becomes keratinized, contains about 9 μmol/g of this dipeptide (Peterson et al., 1983). In vaginal tissue the presence of 4 μmol of the dipeptide/g seems to be low. However, this estimation was carried out in total vaginal tissue and not in keratinized cells alone as in the case of stratum corneum cells.

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ation and identification of *Micrococcus roseus* and *Planococcus* sp. n Schirmacher oasis, Antarctica

SISINTHY SHIVAJI, N. SHYAMALA RAO, L. SAISREE,
VIPULA SHETH, G. S. N. REDDY and PUSHPA M. BHARGAVA
Centre for Cellular and Molecular Biology, Hyderabad 500 007, India

Abstract. Five cultures isolated from soil samples collected in Schirmacher oasis, Antarctica, have been identified as members of the family *Micrococcaceae*, with 3 belonging to the genus *Micrococcus* and two to *Planococcus*. The 3 *Micrococcus* isolates (37R, 45R and 49R) were red-pigmented and had ~75 mol% G+C in their DNA; they were identified as *Micrococcus roseus*. The two *Planococcus* isolates (30Y and Lz3OR) were yellow and orange in colour, and had 43·5 and 40·9 mol% G+C in their DNA respectively; they were identified as *Planococcus* sp.

Keywords. *Micrococcus*; *Planococcus*; taxonomy; Schirmacher; Antarctica.

oduction

robiological studies in continental Antarctica are comparatively few and mostly ined to the Victoria dry valley regions and the McMurdo station area (Madden ., 1979; Johnson and Bellinoff, 1981; Johnson *et al.*, 1981). These studies reveal the most dominant bacteria in the soils of the dry valleys of Antarctica are *robacter*, *Brevibacterium*, *Corynebacterium* and *Micrococcus*. As yet, there are eports on the taxonomy of bacteria present in the oasis regions of continental arctica. The oasis regions, such as the Schirmacher and Bunger oases, are jue in that they are as cold as the dry valleys but differ from the dry valleys in they are under ice cover only during the Antarctic winter, and also experience ificant precipitation (Walton, 1983). It therefore seemed possible that the estrial biology of the oases may vary from that of the dry valley regions. This er highlights characteristics of a group of 5 Gram-positive nonmotile coccoid eria identified as belonging to the genera *Micrococcus* and *Planococcus*.

erials and methods

samples were collected at random sites around lake Zub, Schirmacher oasis 45′12″S and 11°46′E), Antarctica, in the third week of January 1985. The soil peratures varied from +6°C to −6°C.

all the cases 0·5 cm of the surface layer was cleared with a sterile spatula and underlying soil collected and plated after serial dilution on preformed plates aining 0·5% peptone, 0·1% yeast extract, 1·5% agar and 5% (v/v) soil extract Schirmacher oasis. The plates were incubated at 10°C and colony counts were rmined after 7 days of incubation. The optimum temperature and pH for vth of the cultures were determined and the cultures were grown under the

supplementing the plates with appropriate concentrations of NaCl (0·5, 0·1 and 1·5 M).

Cultures in the log phase of growth were observed under the phase contrast microscope for cell shape and size. Motility was determined by direct observation of an overnight culture grown in liquid medium by the hanging drop method and by the piercing of soft agar medium. The presence of flagella was checked by staining the cells by the silver impregnation method (Blenden and Goldberg, 1965).

All tests were performed by growing the cultures at 20°C in the appropriate media. The activities of catalase, oxidase, phosphatase, gelatinase, urease, arginine dihydrolase and β-galactosidase were determined according to standard methods (Holding and Collee, 1971). Production of indole, utilization of citrate, reduction of nitrate to nitrite, and hydrolysis of starch, Tween 80 and esculin were measured following procedures described earlier (Stainer et al., 1966; Holding and Collee, 1971; Stolp and Gadkari, 1981).

Twenty-six different carbon compounds were used to check the ability of the cultures to utilize a carbon compound, provided as the sole carbon source using minimal A medium without glucose (Miller, 1977) but containing 0·2% (w/v) of the carbon source. The ability to ferment a particular carbohydrate, leading to the formation of acid with or without visible production of gas, was monitored according to Hugh and Leifson (1953).

The sensitivity of the cultures to 17 different antibiotics was carried out using HiMedia antibiotic discs or by supplementing the growth medium with the appropriate concentration of the antibiotic.

DNA was isolated from 1 g (wet weight) of cells according to the procedure of Marmur (1961) and the mol% G+C of the DNA was determined from the melting point (T_m) curves obtained using a Beckman 5260 spectrophotometer. The equation of Schildkraut and Lifson (1965) was used to calculate the mol% G+C of the DNA.

Cell walls were isolated and purified according to the method of Work (1971) and analysed after acid hydrolysis for amino acids in a Beckman analyser.

Results

Bacteria were present in all the soil samples; the bacterial count ranged from $0·5 \times 10^3$ to 15×10^3 cells/g of soil (table 1). From the original plates, about 200 colonies were transferred to fresh plates. Out of these, on the basis of colony

Table 1. Bacterial counts in the soils of Schirmacher oasis, Antarctica.

Sample no.	Sample description	Depth of collection (cm)	Colonies x 10^3/g soil	Isolate no.*
37	Soil from lake shore	3	7	37R
45	Soil from lake shore	3	0·53	45R
49	Soil from lake shore	3	1·15	49R

morphology, 45 pure cultures of bacteria were established. The pure cultures consisted mostly of rod-shaped or coccoid bacteria; a few appeared either like long filaments or like chains of bacilli.

Morphology

Of the 45 pure cultures, 5 cultures, namely 37R, 45R, 49R, 30Y and Lz3OR, were selected for detailed taxonomic studies (table 1). All the cultures were Gram-positive, nonmotile, coccoid and pigmented. Cultures 37R, 45R and 49R were red, 30Y yellow, and Lz3OR orange in colour. All the colonies were circular and convex and had a smooth margin; their diameter varied from 1–4 mm. Each individual cell was spherical in shape (1–2 μm in diameter) and lacked flagellum; the cells were present as pairs, tetrads or clusters of cocci.

All the cultures exhibited optimum growth at 20°C; at 5°C, 10°C and 25°C, the growth was slower. At 30°C, only 30Y and Lz3OR could grow (table 2). None of the cultures could grow at 37°C. The optimum pH for growth was 6·9; at pH 4, none of the cultures grew. None of the cultures required NaCl for growth. However, they could tolerate up to 0·5 M of NaCl in the growth medium. At concentrations higher than 1 M NaCl, growth was not observed. Under optimum growth conditions, the generation times ranged from 4·5 (30Y) to 20·37 h (45R).

Table 2. Growth characteristics of *M. roseus* and *Planococcus* sp. from Schirmacher oasis, Antarctica.

	M. roseus			*Planococcus* sp.	
Conditions	37R	45R	49R	30Y	Lz3OR
Temperature (°C)*					
5	+	+	+	+	+
15	+ +	+ +	+ +	+ +	+ +
20	+ + +	+ + +	+ + +	+ + +	+ + +
25	+	+	+	+	+
30	−	−	−	+	+
37	−	−	−	−	−
pH*					
4·0	−	−	−	−	−
6·0	+	−	−	−	+
6·9	+ + +	+ + +	+ + +	+ + +	+ + +
9·0	+ +	+ +	+ +	+ +	+ +
[NaCl]* (M)					
0·5	+ + +	+ + +	+ + +	+ + +	+ + +
1·0, 1·5	−	−	−	−	−
Growth on*					
Citrate agar	+ + +	+ + +	+ + +	+ + +	+ + +
Furazolidone agar	+ + +	+ + +	+ + +	−	−
Acid from aerobic					
Glucose or fructose	yes	yes	yes	yes	yes

Nutrient requirements

The cultures could grow when L-arabinose, D-xylose, raffinose, glucose, D-fructose, D-mannose, D-galactose, sucrose, D-maltose, mannose, lactose, lactic acid, mannitol, glycerol, myo-inositol, sorbitol, citrate, acetate, pyruvate, pyruvic acid, glutamate, formate, malic acid, dextrin, starch or glucosamine were provided as the sole carbon source. None of the cultures produced gas in the presence of any of the 6 carbohydrates used. However, all the cultures acidified the medium in the presence of certain sugars such as glucose and fructose, but not in the presence of others such as sucrose, galactose, mannose and lactose (table 2).

Biochemical characteristics

The biochemical characteristics of the cultures and their response to 17 different antibiotics is shown in table 3. Amino acid analysis of the purified cell walls indicated the presence of Ala, Glu, Lys, Gly and Asp in all the isolates. In addition, the red isolates 37R, 45R and 49R also showed the presence of Ser and Thr. For the preparation of DNA, the cultures could not be directly lysed with sodium dodecyl sulphate (SDS); hence they were treated with lysozyme (for 2–3 h at 25°C) prior to lysis with SDS. The mol% G+C ranged from 41–80. Batch-to-batch variation in the T_m values of the DNA preparations was ±2°C.

Table 3. Biochemical characteristics of *M. roseus* and *Planococcus* sp.

Characteristics	*M. roseus*			*Planococcus* sp.	
	37R	45R	49R	30Y	Lz3OR
Catalase	+	+	+	+	+
Oxidase	−	−	−	−	−
Gelatinase	+	+	+	+	+
Phosphatase	+	+	+	−	−
Urease	−	−	−	−	−
Arginine dihydrolase	−	−	−	−	−
β-Galactosidase	−	−	−	+	+
Indole	−	−	−	−	−
Nitrate reduction	+	+	+	−	−
Hydrolysis of esculin, starch, Tween 80	+	+	+	+	+
Lysozyme susceptibility	+	+	+	+	+
Sensitivity to Kanamycin, streptomycin, erythromycin, novobiocin, neomycin, penicillin G, vancomycin, polymyxin-B, tetracycline, chloramphenicol, ampicillin, nitrofurantoin, gentamycin and rifamycin	S	S	S	S	S

Discussion

To the best of our knowledge, this is the first report on bacteria from an oasis region of Antarctica. The 5 isolates reported in this paper had all the main features of bacteria belonging to the family *Micrococcaceae* (Schleifer *et al.*, 1981; Schleifer, 1984). This family consists of 4 genera, namely *Micrococcus, Stomatococcus, Planococcus* and *Staphylococcus*, which can be differentiated on the basis of their morphology, physiological characteristics, cell wall composition and mol% $G+C$ of DNA (Schleifer, 1984). Based on these criteria, 37R, 45R and 49R, which form irregular clusters in liquid medium, are nonmotile, are capable of growth on furazolidone, do not ferment glucose, and have a mol% $G+C$ of DNA ranging from 73–80%, have been identified as belonging to the genus *Micrococcus* (Schleifer *et al.*, 1981; Kocur, 1984a). The remaining two isolates (30Y and Lz3OR) also formed irregular clusters but differ from the above isolates in that they are incapable of growth on furazolidone agar and have a very low $G+C$ content (41%). Based on these specialized characteristics, isolates 30Y and Lz3OR have been assigned to the genus *Planococcus* (Kocur, 1984b).

A species-level identification of all 5 isolates was attempted based on the characteristics published for the type cultures (Kocur and Schleifer, 1981; Schleifer *et al.*, 1981; Kocur, 1984a, b). Isolates 37R, 45R and 49R, which are red in colour, nonmotile, produce acid from glucose, reduce nitrate to nitrite, grow on glutamic acid as carbon, nitrogen and energy source, and have mol% $G+C$ of DNA ranging from 66–75%, have been identified as *M. roseus*. An earlier study by Johnson *et al.* (1981) had identified, in addition to *M. roseus*, *M. luteus* and *M. freudenreichii* in the soils of the dry valleys of Antarctica. The present isolates resemble *M. roseus* from the dry valleys in having similar maximum temperature (25–30°C) and pH (9–10) for growth, a high mol% $G+C$ of DNA (68–75), and lysine as the diamino acid in the cell wall. Johnson *et al.* (1981) had, however, not studied the other biochemical characteristics of the *Micrococcus* isolates.

Two distinct groups have been identified in *Planococcus*: all strains with 39·5–42·2% $G+C$ in DNA fall into one group, and the remaining, with 47–51% $G+C$, into another group. This second group includes two species, *P. citreus* and *P. halophilus*. Our isolates 30Y and Lz3OR which have a low $G+C$ content (41–43%) and are incapable of growing in agar containing 12% NaCl, do not belong to these two species but could be assigned to the other group. Strains belonging to this group (with mol% $G+C$ in the range 39·5–42·2) bear no species name and have been tentatively designated as *Planococcus* sp. (Kocur and Schleifer, 1981; Kocur, 1984b). Further, isolates 30Y and Lz3OR, unlike other species of *Planococcus*, are not motile and do not possess a flagellum. Miller and Leschine (1984) have reported the presence of a *Planococcus* in the dry valley soils of Antarctica that was also nonmotile and did not resemble any of the known species. The present isolates closely resemble this earlier isolate in that they are psychrophilic, halotolerant, yellow to orange in colour, Gram-positive, nonmotile, non-sporulating, strictly aerobic, and oxidase- and phosphatase-negative (Miller and Leschine, 1984).

The medium normally used for enrichment of *Micrococcus* and *Planococcus* is

by Miller and Leschine (1984) that *Planococcus* from the dry valleys of Antarctica show very little growth in the presence of 1·5 M NaCl.

The present isolates of *M. roseus* and *Planococcus* sp. do not identify completely with the respective type strains in that they cannot grow at 37°C or in the presence of 1 M NaCl; they also could hydrolyse starch, esculin and Tween 80. However, at least two other species of *Micrococcus* are capable of hydrolysing esculin, starch and Tween 80 (Schleifer *et al.*, 1981; Kocur, 1984a). These differences between the Antarctic isolates and the mesophilic type strains may reflect the psychrophilic nature of the Antarctic bacteria and their adaptation to the prevailing climatic conditions. Isolates of *Chromobacterium lividium* (Wynn-Williams, 1983) *Halomonas subglaciescola* (Franzmann *et al.*, 1987), *Flectobacillus glomeratus* (McGuire *et al.*, 1987), *Desulfovibrio* sp. (Rees *et al.*, 1986) and *Flavobacterium acquatile* (Tearle and Richard, 1987) from Antarctica have also been shown to have atypical characteristics and do not identify with the type strains. The present study shows, for the first time, the presence of *M. roseus* and *Planococcus* sp. in an oasis region of Antarctica.

Acknowledgement

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sive monogenic mutation in grain pea (*Pisum sativum*) that pyridoxine requirement for growth and seed production

SUSHIL KUMAR

Biotechnology Centre, Indian Agricultural Research Institute, New Delhi 110 012, India

Abstract. A stable pyridoxine-deficient pea mutant was obtained by screening the M2 progeny of azide-treated *Pisum sativum* cv Pusa Harbhajan. The mutation is visible lethal. The isolation of pyridoxine-deficient mutant demonstrates directly that pea plants synthesize their own pyridoxine and that pyridoxine is an essential growth factor for pea plants. The mutant character is determined by homozygous recessive alleles, designated *pdx-1*, at a single locus. Pyridoxine-deficient plants are fertile and indistinguishable from the wild type if supplied exogenously with 2 mg of pyridoxine.

Keywords. *Pisum sativum* mutant; pyridoxine auxotroph; pyridoxineless pea mutant; inheritance of pyridoxineless mutation; pyridoxine-requiring plant mutant.

ction

isum sativum, dicot, $2n = 14$) is a grain legume crop plant of world-wide ance. It is also one of the most intensively studied plants in physiological, nical, genetic, molecular biological and breeding experiments. Pea has received on as an experimental plant largely because it has large flowers, a self- ting sexual mechanism and a wide array of easily observable seed, seedling lult plant phenotypes (Murfet, 1985). Several genetic markers have been l on each of its 7 chromosomes (Blixt, 1974). These criteria make pea a useful model for molecular genetic analysis of biosynthetic and developmental ses and organization and regulation of genes, and for developing new al for breeding superior varieties of pea and other crop plants. In this context kinds of biochemical gene markers are required in pea to provide basis for tions of methods of molecular genetics (genetic engineering).

ar, two kinds of fertile biochemical (conditional lethal) mutants have been d in pea. These are the chlorate-resistant, nitrate reductase deficient mutants r *et al.*, 1982) and thiamine-deficient mutants (auxotrophs) (Kumar and a, 1986). The available thiamine auxotrophs (Thi⁻) belong to 3 complemen- groups, analogous to *thiA*, *thiB* and *thiC* genes of *Escherichia coli* (Kumar harma, 1986). A third kind of conditional lethal marker in pea, namely a xine (Pdx) mutant, in which auxotrophy is controlled by a pair of recessive (*pdx-1*) at a single locus is reported here. This is the first report of Pdx ophy in higher organisms. The genetic control of Pdx synthesis has been l well only in *E. coli*.

plants were grown to maturity and single plant seeds were harvested. A to
552 M$_2$ progenies that became available were examined for abnormal pheno
from seedling emergence stage to maturity and thus a Pdx$^-$ mutant was iden
The mutant was multiplied and tested in subsequent seasons. Reciprocal cr
Pdx$^-$ × wild type were made and studied to understand the inheritance o
mutation.

Results and discussion

Of the 552 M$_2$ progenies examined, two segregated for Thi deficiency muta
Within a third M$_2$ progeny there was one plant which also had visible
phenotype like that of the Thi auxotrophs. It grew and produced 8 green leave
a normal plant. Later it produced 3 yellowish leaves and a flower and sto
growing. The phenotype strongly resembled that of Thi mutants, but the
failed to respond to application of Thi pyrophosphate. When a mixture of vita
was applied, the plant resumed growth and produced a number of green leave
flowers. A few flowers matured into seed-filled pods. Seeds were collected fro
mutant plant and from the normal-looking plants of the M$_2$ line among whic
mutant was found. They were sown separately to obtain M$_3$ generation prog
The seeds from the mutant plant produced identical plants. Mutant plants
also recovered as segregants in some M$_3$ progenies. After the mutant M$_3$ plant
started to produce yellow leaves, criss-cross pools made with 2 mg/ml of
hydrochloride, Thi hydrochloride, riboflavine, biotin, nicotinic acid, myo-ino
calcium pantothenate, folic acid and p-aminobenzoic acid were applied t
plants. Yellowing and arrest of growth were found to be suppressed by those
that contained pyridoxine hydrochloride. Later it was found that applicati
about 2 mg of Pdx hydrochloride alone could cure the mutant plants of
deficiency symptoms. The mutant displayed a homozygous genotype by bre
true in M$_4$ and M$_5$ generations. Thus it was concluded that the mutant fail
synthesize Pdx and its initial normal growth was due to Pdx received b
developing embryos from the mother plant through placental tissue. It was f
that the onset of yellowing in mutant plants correlated with the amount o
applied to their mother plants. Mutant plants exhibited very tight phenotype
their mother plants had been applied about 100 μg of Pdx hydrochlorid
mutant plants having tight phenotype yellowing started in any of the first 5 leav
was also observed that the yellow leaves of mutant plants had strikingly nar
leaflets compared to those on normal leaves.

Genetic behaviour of the mutation was studied by making reciprocal cr
between the mutant and the parent wild-type plants. Table 1 gives the resu
crosses. It was observed that (i) all the F$_1$ hybrid progeny plants had wild
phenotype, and (ii) F$_2$ seedlings segregated for the wild type and m
phenotypes in 3:1 ratio. The results indicated that the Pdx auxotrophy mu
controlled by a single recessive gene. The allele has been designated $pdx-1$.

Table 1. Segregation of the *pdx-1* mutation in pea.

Genotypic description of cross	Number of plants		Expected ratio	χ^2	P
	Pdx^+	Pdx^-			
F_1 ($+/pdx-1$)	25	$\cdot$0			
F_1 ($pdx-1/+$)	22	0			
F_2 ($+/pdx-1$)	219	67	3:1	0·09	0·95–0·50
F_2 ($pdx-1/+$)	110	38	3:1	0·04	0·95–0·50

ere are a number of implications of the present work, including the following.
has been directly demonstrated that plants require Pdx for their growth and
make their own Pdx. Assessment of the dependence of plant growth and
st index on Pdx synthetic ability will provide criteria for selection in breeding
iments of genotypes having optimum Pdx synthetic capability. (ii) The *in vitro*
red cells, tissues and organs and gynoecia of *pdx-1* mutant plants can be
oyed as recipients for transfer of Pdx^+ linked genes of homologous or
ologous origin so that the rare recombinants could be selected under
ctive conditions. (iii) It will be possible to study and manipulate Pdx synthesis,
ption and transport and roles of Pdx in metabolism. (iv) Knowledge about
henotype of Pdx⁻ mutant will permit isolation of more Pdx⁻ mutants in pea
ther plants.

sides pea, Pdx⁻ mutants are known in *E. coli* (Bachmann, 1983), *Salmonella*
murium (Sanderson, 1984) and *Neurospora crassa* (Perkins et al., 1982). In
li, mutations in any of 5 different genes cause Pdx auxotrophy (Bachmann,
. The available information on bacteria, protozoa, fungi, and invertebrate and
orate animals indicate that Pdx is associated as a cofactor with a number of
nes, some of which are known to be involved in the synthesis of several amino
(Sauberlich, 1968; Snell and Haskell, 1971).

hough auxotrophs for essential vitamins, amino acids and nitrogenous bases
cleic acids are among the most frequently isolated mutations in prokaryotes
ower eukaryotes, a very small number of such mutants have been isolated in
s (McCourt and Somerville, 1987; Last and Fink, 1988; Reddy and Kumar,
. The currently available tight fertile auxotrophs in crop plants of food value
i) the Thi-requiring mutants in tomato, *Lycopersicon esculentum* (Boynton,
, b); grain pea, *Pisum sativum* (Kumar and Sharma, 1986); and barley,
eum vulgare (Kumar and Sharma, 1987; Reddy et al., 1988); (ii) the proline
trophs in corn, *Zea mays* (Racchi et al., 1981); and (iii) the Pdx-requiring
nt of grain pea (present study).

conclusion, a Pdx⁻ mutant of *P. sativum*, designated *pdx-1* has been isolated.
pdx-1 is a single-locus, recessive mutation inherited according to Mendelian
gation. The lethal phenotype of the mutant demonstrates that in plants Pdx is
tial for growth. The mutant *pdx-1* provides a new system for studies in plant
cular biology.

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ction and *in vivo* growth inhibition of Ehrlich ascites tumor cells
alin

H. AHMED, B. P. CHATTERJEE* and A. K. DEBNATH†

Department of Biological Chemistry, Indian Association for the Cultivation of Science, Jadavpur, Calcutta 700 032, India

†Present address: Pomona College, Seaver Chemistry Laboratory, Clarmont, California 91711, USA

Abstract. Jacalin has been found to agglutinate Ehrlich ascites cells. The agglutination was inhibited by α-glycosides of D-Gal and β-D-Gal-(1→3)-D-GalNAc suggesting that the lectin–ascites interaction was carbohydrate-specific. There was 21·8% inhibition of tumour (ascites) cell growth *in vivo* in mice administered 50 μg of jacalin by injection for 6 days following intraperitoneal injection of ascites cells. Administration of 100, 150 and 200 μg jacalin resulted in 40·2, 57·5 and 83% inhibition respectively. The *in vivo* inhibition of tumour cells growth by jacalin was due to its preferential binding with D-Gal-α-(1→6) present as terminal residues in the glycoprotein on tumour cell surface.

Keywords. Ehrlich ascites cells; jacalin; jackfruit lectin; *Griffonia simplicifolia*; lectin.

ction

rlich ascites tumour cell is a spontaneous murine mammary adenocarci-
Ehrlich and Apolant, 1905) adapted to ascites form (Loewenthal and Jahn, d carried in outbred mice by serial intraperitoneal (i.p.) passage. The strong ion of Ehrlich ascites cells (EAC) with plant lectins from castor bean . et al., 1970) and red kidney bean (Nachbar et al., 1976) has been described. ctins from wheat germ, lentil, pea, broadbean, soybean and potato ated the cells moderately, while those from *Lotus tetragonolobus*, *Griffonia* folia II (GS II), *Canavalia ensiformis*, *Helix pomatia*, *Dolichos biflorus* and *us lunatus* either weakly agglutinated the cells or did not (Eckhardt and in, 1983a). The interaction of an α-D-galactosyl-binding lectin from GS I AC and inhibition of their growth by the same lectin were also reported dt et al., 1982). Recently, two other α-D-galactosyl-specific lectins, jacalin ckfruit, *Artocarpus integrifolia* (Suresh Kumar et al., 1982; Ahmed and jee, 1986; Roque-Barreira et al., 1986; Hagiwara et al., 1988), and a lectin *tocarpus lakoocha* (Chowdhury et al., 1987), were shown to agglutinate the lls. The agglutination by *A. lakoocha* lectin was specifically inhibited by α- les of D-galactose, N-acetyl-D-galactosamine and β-D-Gal-(1→3)-D- or its conjugate, β-D-Gal-(1→3)-α-D-GalNAc-O-(CH$_2$)$_2$-NHCO-(CH$_2$)$_7$- H$_3$ (Ahmed and Chatterjee, 1988; Chatterjee et al., 1988).

is paper we report results which demonstrate the *in vivo* cytotoxic effect of on Ehrlich ascites tumour cells.

Materials and methods

All sugars tested and pronase (from *Streptomyces griseus*) were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Neuraminidase (from *Vibrio cholerae*) was the product of Behringwerke AG, Marburg, FRG. β-D-Gal-(1→3)-α-D-GalNAc-O-$(CH_2)_2$-NHCO-$(CH_2)_7$-$COOCH_3$ was obtained by the kind courtesy of Prof. G. Uhlenbruck, Medical University Clinic, Cologne, FRG.

Preparation of jacalin

Fresh seeds of jackfruit were collected from the local market and the lectin (JFL) from these was purified by affinity chromatography on a melibiose–agarose adsorbent as described previously (Ahmed and Chatterjee, 1986).

Preparation of tumour cells

EAC collected from donor mice (Swiss albino) of 18–20 g body weight of either sex were suspended in sterile isotonic saline. A fixed number of viable cells (usually 2×10^6 cells/20 g body weight) were implanted into the periotoneal cavity of each recipient mouse. The tumour cells multiplied relatively freely within the peritoneal cavity. The cells were withdrawn by sterile disposable syringe and diluted with physiological saline. The viability of the cells was 99% as judged by trypan blue exclusion assay.

Enzyme treatment of cells

Prior to enzyme treatment the cells were washed thrice with 0·15 M NaCl and suspended at a final concentration of 1×10^6 cells/ml. The cell suspension (2 ml) containing 0·2 ml of packed cells was separately incubated with 2 mg of pronase (activity 4 units/mg) and with 50 units of neuraminidase (activity 500 units/ml) respectively for 30 min at 37°C. After incubation the enzyme-treated cells were washed thrice with saline, resuspended and preserved at 4°C.

Agglutination and agglutination-inhibition assays

Agglutination assays were preformed in Takatsy microtitre plate according to Chatterjee *et al.* (1979). To 2-fold serial dilutions of lectin solution (25 μl) in saline were added 25 μl of untreated or enzyme-treated EAC ($1–2 \times 10^6$ cells/ml). After incubation for 1 h at 25°C, the agglutination was recorded under a microscope. The reciprocal of the highest dilution of the lectin giving agglutination was expressed as the titre. The experiments were performed in duplicate and controls were set up using saline instead of the lectin.

Agglutination was also carried out in small tubes containing different amounts of

glutination-inhibition test was carried out as follows. To 2-fold serial
of sugar solutions (25 μl) in saline was added an equal volume of two
ating doses of lectin. The mixture, after incubation for 2 h at 25°C, was
ith 25 μl of EAC (1·3 × 10^6 cells/ml); the mixture was allowed to stand for
e at 25°C. The degree of agglutination was examined and the highest
of the sugar solution that caused inhibition was recorded. Controls were set
scribed above.

experiments

ce were given i.p. injections of EAC (∼2 × 10^6) in 200 μl aliquots per mouse.
growth in each animal was monitored by recording daily weight change of
a Mettler P–163 balance.
groups, each group consisting of 6 mice, were given i.p. injections of EAC
. according to the following schedule. Group 1 received EAC on the first
1), and injections of 50 μg JFL from the second day up to the seventh day.
2, 3 and 4 were treated in the same manner with 100, 150 and 200 μg JFL
ely. Group 5 received EAC on day 1 and the cells were allowed to grow in
up to the seventh day. Group 6 received daily injections of 100 μg lectin
ys. Group 7 received daily injections of 100 μl saline for the same period
used as control.
ectin solution and saline used were sterilized by membrane filtration
a 0·45 μm Millipore filter. The injections were given with sterile disposable
inges with 22 gauge sterile needles.

and discussion

lutinated untreated and enzyme-treated EAC. The minimum concentration
ed JFL required to agglutinate untreated EAC was 8 μg/ml, whereas that
for pronase- and neuraminidase-treated cells was 2 μg/ml and 4 μg/ml
ely. The amount of JFL needed to agglutinate untreated EAC (8 μg/ml)
and 167 times more than that required to agglutinate the same amount of
perm (4 μg/ml of JFL), rat lymphocytes (1 μg/ml) (unpublished results) and
ntreated erythrocytes (48 ng/ml) (Ahmed and Chatterjee, 1986) respectively.
cal to compare the degree of agglutination of EAC by JFL to that by other
FL (8 μg/ml, the minimum amount required to agglutinate EAC) is 20, 6·5
mes less potent than GS-1 B$_4$ (0·4 μg/ml), R. communis lectin (1·23 μg/ml)
aseolus vulgaris lectin (2·66 μg/ml) and 2·2 times more active than wheat
gglutinin (18 μg/ml) (Eckhardt and Goldstein 1983a). The lectin from H.
Concanavalin A, lectin from L. tetragonolobus, GS II and lectin from D.
agglutinate at 102, 135, 182, 275 and 675 μg/ml respectively and are almost
to EAC in comparison to JFL.
1 shows the percentage agglutination of EAC with different amounts of

Table 1. Agglutination of Ehrlich ascites tumour cells by jacalin.

Amount of lectin (μg/100 μl)	No. of free EAC* $\times 10^{-4}$	No. of agglutinated EAC $\times 10^{-4}$	Agglutination (%)
4	4·9	2·1	30
10	4·5	2·5	36
20	1·4	5·6	80
40	1·3	5·7	82
80	1·1	5·9	84
120	0·8	6·2	89
160	0·4	6·6	94
200	0·3	6·7	96

*Counted in a hemocytometer (see Materials and methods).

JFL. The percentage agglutination increased with increasing concentration of the lectin. From the number of molecules of JFL (calculated using Avogadro's principle) and the number of EAC involved in agglutination, the average number of lectin molecules bound per cell can be calculated. We obtained a number of 1.8×10^{14} molecules per cell. However, agglutination is a rather crude and inappropriate method for determining the number of bound molecules, and, further, lectin binding does not necessarily cause agglutination of cells (Burger, 1969).

Table 2 shows the results of agglutination-inhibition experiments. α-Glycosides of D-Gal were good inhibitors while the β-anomer was inactive. The agglutination of EAC by JFL was inhibited strongly by β-D-Gal-$(1\rightarrow3)$-α-D-GalNAc-O-$(CH_2)_2$-NHCO-$(CH_2)_7$-COOCH$_3$ (0·78 mM) and methyl-α-D-galactopyranoside (3·12 mM), and moderately by melibiose (25 mM) and raffinose (50 mM), suggesting that the lectin–ascites interaction was specific for α-glycosides of the sugar. The concentrations of the different sugars required for inhibition of EAC agglutination were almost the same as those required to inhibit the hemagglutination of erythrocytes by jacalin (Ahmed and Chatterjee, 1986).

Table 2. Inhibition of jacalin-induced agglutination of EAC by carbohydrates.

Carbohydrate	Concentration*
D-Galactose	400
Methyl-α-D-galactopyranoside	3·12
Methyl-β-D-galactopyranoside	NI
N-acetyl-D-galactosamine	200
p-Nitrophenyl-α-D-galactopyranoside	6·25
Melibiose	25
Lactose	NI
β-D-Gal-$(1\rightarrow3)$-α-D-GalNAc-O-$(CH_2)_2$- NHCO-$(CH_2)_7$-COOCH$_3$	0·78
Raffinose	50

*Minimum amount of sugar (mM) required to neutralize the effect of two agglutinating doses of lectin.
NI, No inhibition up to 400 mM.
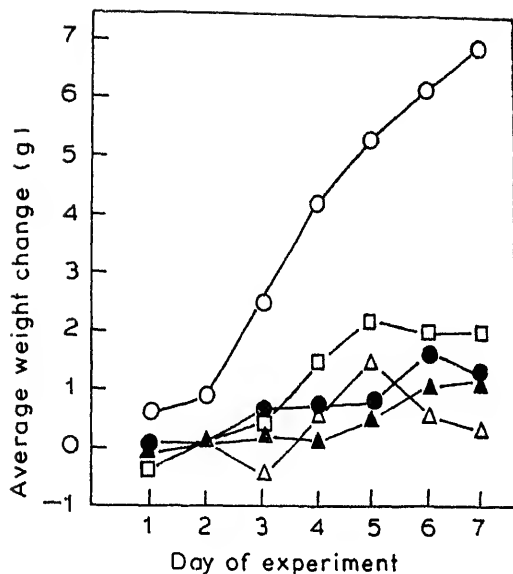
Figure 1. Effect of i.p. administration of jacalin on the growth of EAC *in vivo*. (□), Group 1 (50 μg jacalin + EAC); (▲), group 4 (200 μg jacalin + EAC); (○), group 5 (EAC only); (●), group 6 (100 μg jacalin only); (△), group 7 (100 μl saline only).

en an injection of EAC only had an average weight gain of 6·9 g one week culation. This increase in body weight was due to accumulation of ascites EAC. The control group mice showed a weight gain of 0·3 g after one week. , mice receiving injections of EAC and daily injections of JFL (50, 100, 150 μg respectively) showed a weight gain of 1·2–2 g after the same period.

hibition of EAC growth by JFL *in vivo* is shown in table 3. Mice that daily injections of 50 μg of JFL from the second day after EAC ration showed 21·8% cell growth inhibition. The extent of inhibition was n mice that received daily injections of 100 μg (40·2%), 150 μg (57·5%) and 83·0%) of JFL respectively. By extrapolation of a plot of percentage n *vs* amount of JFL administered it was found that atleast 248 μg of JFL iired for complete inhibition of tumour cell growth.

rdt and Goldstein (1983b) determined the structure of a glycopeptide from

nhibition^a of EAC growth by jacalin.

FL injection	No. of cells in lectin-treated mice (per ml fluid) ($\times 10^6$)	No. of cells in lectin-untreated mice (per ml fluid)	Cell growth inhibition (%)
AC followed by 50 μg JFL/day	140		21·8

EAC plasma membrane which contains an α-D-galactosyl unit in the nonreducing end linked 1, 3 and 1, 6 to a galactose residue. They suggested that GS I lectin reacted with Ehrlich tumour cells and stimulated macrophages, bringing them into close proximity by bridging the α-D-galactosyl groups of their respective glycoproteins (Eckhardt *et al.*, 1982) and causing inhibition of tumour cell growth. JFL agglutinated mouse ascites cells possibly by binding to the α-D-galactosyl residues present in the terminal nonreducing positions of EAC plasma membrane glycoproteins. However, the lower affinity of JFL towards EAC, as judged by its agglutinating ability (8 μg/ml) compared to that of GS I (0·4 μg/ml) (Eckhardt and Goldstein, 1983a), is probably due to the fact that JFL binds only α-D-Gal-(1→6) and not α-D-Gal-(1→3) residues (Ahmed and Chatterjee, 1988) while GS I binds to terminal galactose units with either type of glycosidic linkage (Hayes and Goldstein, 1974).

Acknowledgements

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